

This thesis is dedicated to

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## SUMMARY

Blood transfusion enhances graft survival in renal transplant recipients. The mechanism involved is unclear, but suppression of cell-mediated immunity plays a central role. This has led to the suggestion that blood transfusion may cause immunosuppression in cancer patients and this could have a detrimental effect on their survival.

A retrospective study of 114 patients who had blood transfusion for colorectal cancer was performed. Transfused patients had a poorer 5-year survival rate, but this was probably related to the fact that over 90% of patients with low rectal cancer had an associated peritoneal tumour which was treated. Subsequently, the patients who had abdominal-peritoneal tumours were excluded to limit analysis to the 152 patients who had colorectal cancer. The 5-year survival rate was 47% in the transfused group and 49% in the non-transfused group.

## BLOOD TRANSFUSION AND COLORECTAL CANCER

In the experimental research the aim was to establish a model of colorectal cancer in which blood transfusion could be demonstrated. Analysis of patients with colorectal cancer has been used to assess suppression of cell-mediated immunity. Subjects were treated using fine cytometry and monoclonal antibodies to T lymphocytes. There were no significant changes in subsets after transfusion with blood in animal rats. However, in other rats transfusion with blood caused a significant increase in the number of T lymphocytes.

WILLIAM BEATTIE ROSS

Blood transfusion enhances growth of transplanted tumours in animals and recent evidence implicated macrophage prostaglandin E<sub>2</sub> as a mediator of transfusion-related immunosuppression. The main objective was to study the influence of blood transfusion on the growth of colorectal carcinoma and the possible mediating effects of prostaglandin. This study confirmed that blood transfusion enhanced tumour growth in animals and suggested that treated rats with indomethacin, a prostaglandin synthetase inhibitor, at the time of transfusion may negate this effect.

Using a rat peritoneal macrophage model, it was shown that blood transfusion increased prostaglandin E<sub>2</sub> production by these cells in vitro. Allogeneic serum caused the greatest increase, while transfusion with stored allogeneic and syngeneic blood also caused an increase in prostaglandin E<sub>2</sub> production. This suggests that modulation by factors associated with storage of blood in addition to a genetic effect.

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## ABSTRACT OF THE AUTHOR, WILLIAM B. WASS

Blood transfusion enhances graft survival in renal transplant recipients. The mechanisms involved are unclear, but suppression of cell-mediated immunity plays a central role. This has led to the suggestion that blood transfusion may cause immunosuppression in cancer patients and this could have a detrimental effect on their survival.

A retrospective study of 314 patients who had bowel resection for colorectal cancer was performed. Transfused patients had a poorer five year survival rate, but this was probably related to the fact that over 90% of patients with low rectal carcinoma who had an abdomino-perineal resection were transfused. Subsequently, the patients who had abdomino-perineal resections were excluded to limit analysis to the 159 patients who had abdominal resections. The 5-year survival was 34% in those transfused, compared with 47% in those who were not, but this was not a significant difference.

In the experimental research the aim was to establish a model of colonic cancer in which blood transfusion caused immunosuppression. Analysis of peripheral T cell subsets has been used to assess suppression of cell-mediated immunity. Subsets were counted using flow cytometry and monoclonal antibodies to T lymphocytes. There were no significant changes in subsets after transfusion with blood in normal rats. However, in older rats, transfusion with blood caused a significant increase in suppressor cells.

Blood transfusion enhances growth of transplanted tumours in animals and recent evidence implicated macrophage prostaglandin E2 as a mediator of transfusion-mediated immunosuppression. The next objective was to study the influence of blood transfusion on the course of colonic carcinogenesis and the possible abrogating effects of indomethacin. This study confirmed that blood transfusion enhanced dimethylhydrazine carcinogenesis and suggested that treated rats with indomethacin, a prostaglandin synthetase inhibitor, at the time of transfusion may abrogate this effect.

Using a rat peritoneal macrophage model, it was shown that blood transfusion increased prostaglandin E2 production by these cells in vitro. Allogeneic serum caused the greatest increase, while transfusion with stored allogeneic and syngeneic blood both caused an increase in prostaglandin E2 production. This suggests immunomodulation by factors associated with storage of blood in addition to a genetic effect.

In conclusion, there is evidence that blood transfusion is associated with reduced survival of colorectal cancer patients, but this may be due to more advanced malignancy in transfused patients. Blood transfusion enhances colonic carcinogenesis in rats and this may be abrogated by indomethacin. Blood transfusion increased prostaglandin E2 production by macrophages and this supports the theory that transfusion associated immunosuppression may be mediated by this effect.

DECLARATION OF THE AUTHOR, WILLIAM B. ROSS

This thesis called "Blood Transfusion and Colorectal Cancer" has been composed by myself. The retrospective clinical study was conducted solely by myself. The experimental work was planned by myself, but carried out with the help of my research technician Janine Beavis. I personally performed every injection of carcinogen. Preparation and aliquoting of the carcinogen dimethylhydrazine and indomethacin were done by myself. The majority of the transfusions and blood sampling were performed by myself. Every postmortem examination was performed by myself. Some of the early lymphocyte separation and labelling with monoclonal antibodies was performed by the author, but the majority of this work was done by Janine Beavis. I was present during some of the flow cytometry sessions. All of the macrophage prostaglandin animal work and the prostaglandin assays were performed by the author. I have seen every histological slide, but the histological interpretations are those reported by Dr I Nawroz, pathologist. All the computing and statistical analysis of the data was performed by myself.

SIGNED

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## PUBLICATIONS

### BLOOD TRANSFUSION AND COLORECTAL CANCER

Material included in this thesis has been published prior to submission of the thesis:

#### 7. Acknowledgements

#### 8. Synopsis

ROSS W B. (1987) Blood transfusion and colorectal cancer. Journal of the Royal College of Surgeons of Edinburgh 1987 32 197-201.

#### 9. Discussion

ROSS W B, BEAVIS M J, SALAMAN J R. (1988) T lymphocyte subpopulations in the Sprague Dawley dimethylhydrazine colonic carcinogenesis model. Surgical Research Communications 1988 4 177-182.

ROSS W B, BEAVIS M J, SALAMAN J R. (1988) Allogeneic blood transfusion and immunosuppression in Sprague Dawley rats; T lymphocyte subset analysis. Surgical Research Communications 1988 4 183-186.

ROSS W B, NAWROZ I, BEAVIS M J, SALAMAN J R. (1990) Does indomethacin reverse blood transfusion induced enhancement of colonic carcinogenesis in rats? Surgical Research Communications 1990 7 107-112.

## BLOOD TRANSFUSION AND COLORECTAL CANCER

The first aim of this research was to test the theory that blood

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immunosuppression. The blood transfusion effect may be initiated by a mechanism involving increased synthesis of prostaglandins by macrophages. This possibility was investigated, and evidence to support this theory was found in this experimental model. Increased prostaglandin synthesis was greater in stored rather than fresh blood irrespective of the histocompatibility of the transfused blood.

## 1. PREFACE

The first aim of this research was to test the theory that blood transfusion has detrimental effects in colorectal cancer. A retrospective clinical study of patients undergoing surgery for colorectal cancer suggested that blood transfusion may be associated with poorer survival. The aim of the experimental work was to establish an animal model in which the effects of blood transfusion on colonic cancer could be studied. It was shown that blood transfusion enhanced colonic carcinogenesis. Enumeration of T cell subsets was used to assess changes in the immune system. In normal rats blood transfusion did not alter these, but in older rats blood transfusion changed the T cell subsets in a manner suggestive of immunosuppression. The blood transfusion effect may be mediated by a mechanism involving increased synthesis of prostaglandins by macrophages. This possibility was investigated, and evidence to support this theory was found in this experimental model. Increased prostaglandin synthesis was greater in stored rather than fresh blood irrespective of the histocompatibility of the transfused blood.

Cardiff Royal Infirmary.

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### 3. SYNOPSIS

Blood transfusion enhances graft survival in renal transplant recipients. The mechanisms involved are unclear, but suppression of cell-mediated immunity may play a central role. This has led to the suggestion that blood transfusion may cause immunosuppression in cancer patients and this could have a detrimental effect on their survival.

A retrospective study of 314 patients who had bowel resection for colorectal cancer was performed. Complete records were retrieved for 302 patients. Of these, 236 had potentially curative resections. In this group, transfused patients had a poorer five year survival rate, but this appeared to be due to a larger number of low rectal tumours treated by abdomino-perineal resections, and a higher incidence of other adverse prognostic variables in the transfused group.

The first aim of the experimental work was to establish a model of colonic cancer in which blood transfusion caused immunosuppression. Analysis of peripheral T cell subsets has been used to assess suppression of cell mediated immunity. The role of T cell subset analysis was studied in the dimethylhydrazine (DMH) colonic carcinogenesis model in rats. Subsets were counted using flow cytometry and monoclonal antibodies to T lymphocytes. Three weekly transfusions were given of allogeneic blood or saline. There were no significant changes in subsets and after transfusion with blood



in normal rats. In older rats, transfusion with blood caused a significant increase in suppressor cells. Subset changes were not seen in rats receiving DMH injections. Helper subsets were higher in rats with advanced malignancy compared with age-matched controls.

Blood transfusion enhances growth of transplanted tumours in animals. The next objective was to study the influence of blood transfusion on the course of colonic carcinogenesis. Sprague Dawley rats were given 16 weekly injections of DMH. During week 8 they were randomly divided into two groups. Group 1 were given three weekly transfusions of blood from DA rats and Group 2 were given saline. All rats were killed at 28 weeks and rats transfused with blood had more large bowel tumours than those transfused with saline. Histological analysis was complicated by the close proximity of individual tumours and alterations to the experimental model were made in subsequent experiments to avoid this problem.

An experiment was designed to examine the influence of blood transfusion on prostaglandin production by rat peritoneal macrophages. Allogeneic blood increased prostaglandin E2 production and this effect was larger in stored blood, but greatest in serum. Transfusion with stored syngeneic blood was also associated with increased prostaglandin synthesis.

Macrophage prostaglandin production may be increased by blood transfusion and this potentially immunosuppressive effect may be reversed by indomethacin. Treating rats with indomethacin, a prostaglandin synthetase inhibitor, at the time of transfusion

probably reverses the enhancing effect on carcinogenesis. This experiment confirmed (by histopathology) that blood transfusion enhanced colon carcinogenesis.

In conclusion, there is evidence that blood transfusion may have a detrimental effect on survival of colorectal cancer patients, although transfused patients seemed to have more advanced disease. Blood transfusion enhances DMH colonic carcinogenesis in rats, but it was not shown that this may be reversed with indomethacin. Allogeneic blood transfusion results in increased prostaglandin E2 production by rat peritoneal macrophages and storage conditions of blood may contribute to this.

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#### 4.1. HISTORY OF BLOOD TRANSFUSION

Blood transfusion dates back to 1490 when Pope Innocent VIII was given blood drained from the veins of a youth. It is said that the Pope died together with three youths who were the blood donors. The first successful blood transfusion is credited to Richard Lower in 1667. Most blood transfusions which followed this ended in fatality. In 1900 Karl Landsteiner discovered blood groups. George Crile performed successful blood transfusion in 1907. However, there were still many deaths resulting from incompatible transfusion. The Rhesus factor was discovered in 1940 by Landsteiner and blood transfusion subsequently became safer. The anticoagulant properties of sodium citrate were recognised in 1914. The concept of the blood bank became a reality around the time of the Spanish Civil War.

## 4.2. BLOOD TRANSFUSION AND TRANSPLANTATION

### 4.2.1. The transfusion "effect"

Patients with chronic renal failure often develop anaemia. This is usually normocytic and normochromic in nature. This type of anaemia is particularly marked when the patients are treated with haemodialysis rather than peritoneal dialysis. Patients with renal failure can be maintained on haemodialysis for many years. Many of these patients will require blood transfusion for symptomatic relief of anaemia. In the early days of dialysis transfusions were given liberally. However, in 1966 Kissemyer-Nielsen and colleagues reported two cases of hyperacute rejection of renal transplants. They found lymphocytotoxic antibodies in the preoperative serum of the recipients (Kissemyer-Nielsen, 1966). It was said that these patients had been "heavily isoimmunised by blood transfusions" and they suggested that the pretransplant transfusions were responsible for the production of the lymphocytotoxic antibodies which were directed against the donor cells. This finding resulted in highly restrictive transfusion policies for potential renal transplant recipients. It also led to a greater use of lymphocyte crossmatches which are so important in renal transplantation today. It was subsequently confirmed that patients waiting for a renal transplant who have preformed lymphocytotoxic antibodies in their serum have a smaller chance of receiving a successful graft (Opelz and Terasaki, 1972). Opelz also studied cytotoxic antibodies in patients who were regularly transfused while on haemodialysis (Opelz et al, 1972).

Patients with no demonstrable cytotoxic antibodies for more than one year before transplantation had a significantly better graft survival than patients who had no cytotoxic antibodies for less than a year prior to transplantation. Cytotoxic antibodies only developed in half of the patients who received regular blood transfusions. They suggested that those patients who did not develop antibodies represented a group of "non-responders" who were more likely to accept a renal graft.

In 1973, Gerhard Opelz and his co-workers reported retrospective studies on a group of 148 renal transplant recipients (Opelz et al, 1973). They were surprised to find that 57 patients with a total of more than ten pre-transplant transfusions had the highest survival compared to patients not transfused (n=25) and transfused patients who received less than ten transfusions of blood (n=66). The one-year graft survival rate for the heavily transfused group was 66% compared with 43% for the patients receiving one to ten transfusions and 29% for non-transfused patients. Several retrospective studies followed and these confirmed the beneficial effect of blood transfusion. There has only been one attempt to compare pretransplant transfusion with no transfusion using a prospective randomised trial protocol (Bucin et al, 1981). This confirmed a beneficial effect associated with transfusion.

#### 4.2.2. Mechanisms

Much research has been directed towards identifying the mechanisms involved in mediating the beneficial effects of blood transfusion on renal graft survival. Fifteen years have passed and the problem has not been solved. Multiple mechanisms may be involved because no one theory adequately explains all the known effects of blood transfusion. Some of this work will be reviewed to illustrate the theories which have been put forward.

##### 4.2.2a. Antiidiotypic antibodies

Blood transfusion may induce donor-specific antibody directed against HLA-DR antigens (antiidiotypic antibodies) on the donor lymphocytes. This would enhance graft survival. Morris et al reported 14 successful renal transplants in the presence of a positive B-lymphocyte cross-match against donor tissue (Morris et al, 1978). These findings do not support the selection theory which suggests that blood transfusion induces alloantibodies and this only allows the patient to receive a kidney which is more likely to be accepted. This means that only non-responders would be selected because of their inability to produce lymphocytotoxic antibodies.

Singal and Joseph (1982) tested sera from renal transplant recipients to study the effects of blood transfusions on the



induction of antibodies directed against recognition sites on T lymphocytes. They found that blood transfusion induced antibodies capable of inhibiting proliferative responses in mixed lymphocyte culture against antigens present on the kidney donor. However, such antigens were not present in patients who had rejected the transplant. This suggested that these antibodies which were induced by transfusion may have been associated with prolonged graft survival. More recently Keown et al (1983) have shown that in a series of eighteen patients who had not previously been transplanted the incidence of cytotoxic antibodies to a T lymphocyte panel was only 2%. These patients did not develop broadly reactive anti-HLA antibodies after transfusion. Within the first week after transfusion there was a significant decrease in the mixed lymphocyte culture response of the recipient after stimulation with donor or third party lymphocytes. However, this effect was transient and levels of response returned to normal two weeks after transfusion. In multiparous females and in patients sensitised by a previous graft which had been rejected, blood transfusion induced antibodies against T and B lymphocytes. In this group cytotoxic activity in mixed lymphocyte culture was increased.

#### 4.2.2b. Non-specific immunosuppression

Non-specific immunosuppression by macrophages has been suggested (Keown and Descamps, 1979). They suggested that endocytosis of damaged red cells from the transfused blood impaired normal mononuclear phagocytic function. They postulated that endocytosis

might generate prostaglandin which could induce suppressor cells. Watson et al (1979) measured cell-mediated immunity by skin testing with dinitrochlorobenzene. The degree of skin reaction to this chemical is a measure of cell-mediated immune function. They found that patients with weak reactions had a higher graft survival after transplantation and that the weak responders had had more pretransplant transfusions. In fact the patients who were weak responders were more anaemic than strong responders so these findings could not support a theory which suggested that blood transfusion caused a general suppression of cell-mediated immunity.

#### 4.2.2c. Lymphocyte responses

Another group assessed cell-mediated immunity by measuring the lymphocyte responses to mitogenic and antigenic stimulation (Fischer et al, 1980). Non-transfused prospective kidney transplant recipients were transfused with washed red cells. They found a reduced lymphocyte response after transfusion. A second transfusion led to a more pronounced and prolonged suppression of this response. Normal volunteers were given transfusions of autologous blood, but no suppression of cell mediated immunity was found.

Kerman et al (1982) assessed cellular immunity by testing spontaneous lymphocyte blastogenesis, skin test antigen responses, lymphocyte response to alloantigen and mononuclear suppressor cell activity. Patients who received more than five pretransplant transfusions were found to be weak immune responders and have a

strong in vitro suppressor cell function. They suggested that these "traits" may explain the beneficial effect of transfusion on graft survival.

#### 4.2.2d. Suppressor cells

A group in Cardiff showed that blood transfusion was associated with increased suppressor cell function in patients undergoing haemodialysis (Smith et al, 1983). This method involved Con-A lymphocyte stimulation before and after cell culture. Suppressive activity is lost during culture, but is retained if suppressor activity is high. Lenhard et al (1982) showed that blood transfusion suppressed the mixed lymphocytic reaction and increased the number of monocytes. The mixed lymphocytic reaction returned to normal after removal of the monocytes. They suggested that monocytes act as suppressor cells. They also found that T cell suppressor activity increased later on in the post-transfusion period suggesting that at least two immunoregulatory mechanisms may be induced by blood transfusion.

Another theory suggests a two stage process (van Rood, 1983). Firstly there is a non-specific suppression of cytotoxic lymphocyte reactivity and induction of anti-HLA class I (A,B,C) antibodies. Then specific anti-HLA antibodies are produced which prevent activation of T lymphocytes by class I and class II (D,DR) in the donor blood. These antibodies may subsequently block host T lymphocyte activation in response to class I antigens on the graft.

This theory explains why transfusion with HLA-matched blood exerts no beneficial effect on graft survival (Albert et al, 1981). It must be noted that HLA typing at that time only allowed classification of class I antigens and HLA-D,DR typing could not be performed on the patients involved.

#### 4.2.2e. Clonal deletion theory

Terasaki (1984) proposed the clonal deletion theory. This suggests that blood transfusion can exert a beneficial effect only in the presence of immunosuppressive therapy. Multiple blood transfusions will sensitise recipients to a wide range of antigen. After transplantation a rapid immune response is directed against previously encountered antigens present on the graft. However, the presence of immunosuppressive therapy at the time of grafting deletes the clones of reactive lymphocytes.

#### 4.2.3. Amount, type, and timing of the transfusion

Do the amount, type and timing of blood transfusion influence the observed beneficial effect? Stiller et al (1978) reported that transfusion given on the day of transplantation improved graft survival in patients who had not been transfused previously and also in patients who had received pretransplant transfusions. This retrospective study also confirmed the beneficial effect of pretransplant transfusion on graft survival at one year (71% for

transfused and 40% for non-transfused). Corry et al (1980) found that transfusions given at the time of surgery had a beneficial effect, but other studies have failed to confirm this (Opelz and Terasaki, 1981). Hourmant et al (1979) demonstrated a small benefit in patients transfused within three months prior to transplantation compared with patients transfused at an earlier stage.

Pretransplant transfusion usually consists of whole blood or packed red cells. Both types of blood improve graft survival. Packed cells possibly exert a greater effect than whole blood and frozen blood preparations do not influence graft outcome (Opelz and Terasaki, 1980). Leucocyte-free blood can be produced by filtering with cotton wool and it does not have an effect on graft survival (Persijn et al, 1979). The same report showed that leucocyte-poor blood (washed red cells) did exert a beneficial effect. Rapaport and Dausset (1983) have demonstrated that blood leucocyte extracts enhance skin allograft survival in humans. The leucocytes express HLA antigens whereas red blood cells do not (the situation differs in rats and mice where histocompatibility antigens are also present on red blood cells).

The benefit on graft survival increases with the number of transfusions given (Opelz and Terasaki, 1978). This has been confirmed by several studies. Opelz reviewed data from 200 transplant centres and was able to study nearly 7000 transplant recipients (Opelz, 1985). Patients who had received six to ten transfusions had the highest one year survival rates. However, patients who had received more than ten transfusions had a lower

graft survival rate.

#### 4.2.4 Live donor renal transplantation

Salvatierra first reported the beneficial effects of donor specific transfusion in live-related donor renal transplants (Salvatierra et al, 1980). This study demonstrated a highly significant survival advantage for transfused recipients of a one HLA-haplotype matched live related donor kidney. Similar effects have been demonstrated by others (Glass et al, 1985). A hazard of donor specific transfusion is sensitisation with the appearance of anti-donor T lymphocyte antibodies which will contraindicate the intended transplantation. This may occur in up to 30% of cases, but anti-donor sensitisation can be abolished by concomitant administration of cyclosporin with donor specific transfusion prior to transplantation; however, donor specific transfusion may be associated with earlier rejection episodes (Sells et al, 1989). The situation is confused by the recent finding that when compared with random donor transfusions, donor specific transfusion may not have a superior beneficial effect (Opelz, 1989). This study also indicated that the transfusion effect present at one year after transplant was not seen at three years i.e. transfused and non-transfused patients had similar long-term graft survival rates.

#### 4.2.5 Transfusion, transplantation and cyclosporin

The latest update on the Collaborative Transplant Study published in 1989 indicates that the influence of pretransplant transfusion on graft survival has been diminishing since 1984 (Opelz, 1989). This study includes 17,000 patients (90% were transfused) and still demonstrates a small significant beneficial effect associated with transfusion, but it would seem that the risks of sensitisation and disease transmission may now outweigh this small advantage (Anonymous, 1990).

#### 4.3. BLOOD TRANSFUSION AND EXPERIMENTAL CANCER

##### 4.3.1. History

Experimental evidence that blood transfusion influences tumour growth dates back to the early 1950's when Snell (1955) reported that blood transfusion enhanced the growth of tumour transplants in mice.

##### 4.3.2. Blood transfusion enhances tumour growth

Previous work had shown that pretreatment with some tissue preparations could produce tolerance of tumour transplants in host mice when these tissues or their extracts were injected intraperitoneally (Snell, 1954; Kandutsch, 1959). The tissue preparations included tumour, spleen and kidney. The tumour used in the experiments was a mouse sarcoma. The recipient mice of the tumour graft were transfused with whole blood from the same strain of mouse used as a source of the sarcoma. Results were expressed as the proportion of mice dying from progressive tumour growth.

Intravenous blood transfusion was the most effective regime and consisted of five transfusions. In this group of mice there were 26/27 deaths. A second group of mice received five intraperitoneal injections of blood: 11/20 deaths occurred. Spleen and renal tissue



preparations, also syngeneic with the sarcoma, did not produce a similar effect. A control group had 0/19 deaths following tumour transplant. Therefore, in this sarcoma transplant model virtually complete tolerance to grafts of sarcoma (and early death) were obtained by donor specific whole blood transfusion. At the time it was known that whole blood could produce immunity to homografts and the authors concluded this paper with the statement, "the factors involved in the production of tolerance instead of immunity in this particular experimental situation are still largely obscure".

Workers from Sapporo, Japan used a rat model to investigate allogeneic blood transfusion and anti-tumour immunity in rats (Oikawa, 1977). Immunisation consisted of one 2 ml intravenous injection of donor blood given 7 days before tumour implantation. The tumour used was a fibrosarcoma induced by a subcutaneous injection of 3-methyl-cholanthrene in syngeneic rats. Inhibition of tumour growth was seen with four strains of allogeneic donor blood. However, some strains were associated with a more potent effect than others. White blood cells from one of the strains were treated with mitomycin C. This process did not appear to alter the inhibitory influence of blood transfusion on tumour growth. This ruled out a graft-versus-host reaction mediating the effect. It had been suggested that inhibition of tumour growth might be due to cross-reactive antigens between immunising cells and challenged tumours. The authors looked for cross-reactive antigens by cytotoxicity and immunofluorescent tests, but found none. They concluded that the mechanism of inhibition of tumour growth may be

due to a non-specific immunostimulation of the host by immunisation with alloantigens.

After Opelz had published data on the beneficial effects of blood transfusion in renal transplant recipients in 1981 and Gantt had suggested that blood transfusion may enhance the growth of tumours in patients with cancer, Francis and Shenton (Newcastle) published a brief report on their animal experiments (Francis, 1981).

They used three groups of five Wistar rats and transfused these rats prior to subcutaneous injection of a tumour homogenate derived from a methylcholanthrene-induced sarcoma. One group was transfused with two 2ml injections of whole blood three days apart, the blood being allogeneic for the recipient strain. The second group received similar transfusion with syngeneic blood while the third group received saline. They measured the growth of the tumour and then excised and weighed the tumour at 14 days. They took blood samples at intervals to measure peripheral blood lymphocyte responses to purified protein derivative of old tuberculin and phytohaemagglutinin. The plasma suppressive activity was also measured (Veitch, 1978).

They reported that plasma suppressive activity was increased and that lymphocyte reactivity was reduced after transfusion with allogeneic blood, but not after syngeneic blood or saline transfusion. The rate of tumour growth and the final weight of the excised tumour were significantly greater in the group transfused with allogeneic blood than in the other two groups. There were no

differences between the group transfused with syngeneic blood and the group transfused with saline.

This work was also reported later elsewhere in abstract form (Francis, 1982a). Francis et al expanded their experimental groups to twelve rats in each and found similar results to those cited above (Francis, 1982b).

Hormini and co-workers (Okayama) investigated the influence of blood transfusion on transplanted tumours in mice (Hormini, 1983). The tumour transplants were a hepatoma originating in C3H/He mice and a lung carcinoma in Lewis mice. Suspensions of cells from these tumours were injected subcutaneously into various strains of mice which were either syngeneic or allogeneic with the strain from which the tumour cells were derived. Blood transfusion consisted of one 0.3 ml intravenous injection of whole blood or saline or various blood components. These blood components were red cells, B lymphocytes, T lymphocytes, thymus cells and plasma. Tumour growth was determined by measurement of the diameters at intervals. They found that some, but not all of the allogeneic transfusions promoted tumour growth in the recipients. Recipients transfused with syngeneic blood showed no increase in tumour growth compared with rats transfused with saline.

Components of allogeneic blood known to have positive effect on tumour growth were then transfused. The red cell only component did not accelerate tumour growth. T lymphocyte, thymus cell and B lymphocyte components greatly increased tumour growth. Plasma was

found to have the most potent effect. These findings are at variance with those of Jeekel. This can be explained partially by the fact that both animals and tumours used were different.

A further series of experiments were reported in a Japanese journal by the same group from Okayama (Kagawa, 1984). However, the English abstract suggests that their findings were very similar to Hormini's results.

Singh, Marquet, Jeekel et al (Rotterdam) used a rat model to investigate the effects of surgery and blood transfusion on the growth of established tumour metastases (Singh, 1987). They used a different tumour model to that previously studied (Jeekel, 1982). This new tumour was a "nonimmunogenic", spontaneous sarcoma in BN rats. The tumour was maintained in cell culture and tumour cell suspensions were injected intravenously. This procedure resulted in pulmonary metastatic deposits which could be counted. The surgery comprised a small bowel resection under ether anaesthesia. Blood transfusion consisted of a single intravenous injection of 1ml of syngeneic or allogeneic blood. This injection was given at either seven days before or seven days after injection with tumour cells.

Syngeneic blood transfusions and surgery (alone or combined) had no effect on tumour growth. However, allogeneic blood transfusion significantly increased the number of lung deposits. This effect was even greater when allogeneic blood transfusion was combined with surgery. However, allogeneic blood did not exert this effect (described as the effect on the "take" of tumour cells) when given

seven days before tumour inoculation. Tumour take was increased by surgery (bowel resection) on the day of tumour inoculation, but surgery alone did not influence the growth of established metastases.

The same group from Rotterdam assessed the effect of a single blood transfusion on the formation of lung metastases in rats (Marquet, 1986). In rats with established metastases, allogeneic transfusion stimulated the growth of these tumour deposits. However, using different strains of rats they found that blood transfusion inhibited the development of new metastases. Further studies by this group demonstrated that surgical trauma increased the enhancing effect of allogeneic blood transfusion on development of lung metastases after injection of sarcoma cells (Marquet et al, 1989).

Clarke and Tarin (Oxford) studied the effect of blood transfusions on tumour metastases using a mouse model (Clarke, 1987). The subcutaneous growth rate and metastatic spread of syngeneic melanoma and fibrosarcoma were assessed. These tumours were maintained as cell lines in monolayer tissue culture. The harvested cell suspensions were injected subcutaneously and spontaneous lung metastases occurred. Blood transfusion consisted of one intravenous injection of 0.25ml whole blood (allogeneic or syngeneic) 14 days prior to tumour inoculation.

Compared with syngeneic blood and saline transfusion, allogeneic blood did not affect the growth rate of primary tumours, but did

increase and accelerate spontaneous metastasis. However, this effect was not observed in one of the four strains of allogeneic blood used in the experimental groups. Similar increases in spontaneous metastases were seen when tumour graft recipients were transfused with allogeneic peripheral blood lymphocytes and splenocytes. This effect was again dependent on the strain of the allogeneic blood donor.

This group reported further experiments which confirmed that the growth of the experimental UV-2237 metastases was augmented by transfusion with certain strains of allogeneic blood given both before and after tumour inoculation (Clarke et al, 1989). Syngeneic blood, saline transfusions (controls), and some strains of allogeneic blood did not cause this effect, however.

Melanoma growth in a mouse model is influenced by blood transfusion (Francis et al, 1987). They used a transplantable B16 melanoma tumour in nude (athymic) mice. Normal mice and nude mice transfused with saline were compared with mice transfused with incompatible allogeneic blood. The growth rates of the transplanted tumours were assessed by measuring the tumour diameter at intervals and by weighing the excised tumour. In athymic mice there were no differences between the three groups. However, in normal immunocompetent mice allogeneic blood transfusion enhanced the rate of tumour engraftment and the tumours were also heavier and larger. The authors concluded that the blood transfusion effect depended on the presence of the thymus gland and that this, by implication, meant that cell-mediated immunity must be involved in the mechanism.

This group also reported in another series of experiments, which also used this model, that tumour weight and volume were increased by transfusion with allogeneic blood (Francis, 1986). This effect was seen only when blood was transfused before tumour cells were implanted.

Waymack and Chance showed that allogeneic, but not syngeneic, transfusions increased the rate of tumour growth in rats implanted with a sarcoma when compared with the control group which had been transfused with saline (Waymack and Chance, 1988). These animals were followed until death, but there were no significant differences between survival times.

Shirwadkar et al, using a fibrosarcoma pulmonary metastases mouse model, found that the number of tumour cells injected influenced the allogeneic transfusion effect compared with syngeneic transfusions and a control transfusion of saline: higher numbers of pulmonary metastases occurred with lower numbers of tumour cells whereas higher numbers inhibited development of lung deposits (Shirwadkar et al, 1990).

Parrott et al, demonstrated that blood transfusion enhanced growth of MC7 sarcoma in rats in a dose dependent manner and also that surgical trauma (sham laparotomy) exerted a synergistic effect on this (Parrott et al, 1990). Allogeneic washed cells and plasma also had similar effects, although they were less marked.



#### 4.3.3. Blood transfusion does not enhance tumour growth

Tsukada (1970) studied the effect of blood or blood component transfusion on the survival of rats transplanted with sarcoma and hepatoma cells. He demonstrated that blood from normal rats had an anti-transplantation effect. The transfused rats survived longer. The effect seemed to be due to leukocytes in the transfused blood. These effects were seen only when blood or blood components were transfused prior to tumour transplantation.

Jeekel and colleagues (Rotterdam) reported further conflicting findings (Jeekel, 1982). They had already investigated the effects of transfusion in a rat heart transplant model and found that donor specific blood transfusion could lead to an indefinite survival of cardiac grafts (BN) in recipient (WAG) rats (Marquet, 1981).

They transplanted two types of tumours in WAG rats (Marquet, 1982). The first was a radiation induced basal cell carcinoma and the second was a chemically induced carcinoma of the duodenum. Both these tumours were induced in BN rats. After subcutaneous implantation of the first tumour the doubling time was 2.5 days while the adenocarcinoma had a doubling time of 14 days. Blood transfusion consisted of a single intravenous injection of 1ml of whole blood given 14 days before tumour implantation. The blood was either syngeneic (WAG) or allogeneic (BN) for the recipient rats. There were six experimental groups each containing eight rats.



Allogeneic (donor specific) blood transfusion caused a slight, but not statistically significant, inhibition of tumour growth in the basal cell carcinoma model. A much stronger inhibitory effect was found in the adenocarcinoma model. Isolated basal cell tumour cells were injected intravenously 14 days after transfusion in a third model. Allogeneic blood transfusion caused a reduction by half of the number of pulmonary metastatic nodules counted three weeks after inoculation. Syngeneic (WAG) blood did not affect transplanted tumour (BN) growth in the recipient (WAG) rats. The authors concluded that allogeneic transfusions can lead to a substantial reduction of tumour growth and that this observation may have important clinical implications. This situation can only be applied to clinical cases where patients have been exposed to blood products prior to developing cancer. This group of patients will include women who have been pregnant. The implication suggested by Jeekel cannot be applied to patients who already have cancer at the time of first exposure to blood transfusion, at operation for example.

There were now several contradictory studies concerning the effect of blood transfusion on tumour growth. A group from Heidelberg attempted to clarify the situation by investigating five tumour models in the rat (three syngeneic, one allogeneic, and one autochthonous) using different transfusion regimes (Zeller, 1986).

The autochthonous tumour was a fibrosarcoma induced by a single 6mg injection of benzo(a)pyrene in Sprague Dawley rats. Third party

blood (Lewis) was transfused according to three regimes:- i) five transfusions before tumour induction, ii) five transfusions after tumour induction and iii) five transfusions before and after tumour induction. A control group were transfused with saline at the same intervals as the latter group. The syngeneic tumours were ependymoma, neurinoma and adenocarcinoma of the stomach from BDIX rats. The blood donors for this group were Sprague Dawley rats. The allogeneic tumours were lymphomas from BDX rats transplanted to Sprague Dawley rats. The take rate, induction time, incidence and growth rate of these tumours were measured. T lymphocyte subsets and lymphocyte stimulation by Con-A were measured. The latter was reduced following blood transfusion, but no change was observed in the peripheral T lymphocyte subpopulations.

Allogeneic or syngeneic blood transfusion did not influence tumour behaviour in the transplant tumour models. Allogeneic transfusion did not have an effect on the autochthonous chemically induced fibrosarcoma. This series of experiments involved large numbers of animals and the results are impressive. However, it should be noted that the transfusion protocols included five or ten transfusions. Possibly these were administered under general anaesthesia or some form of sedation. These and the general trauma of the procedures may have been enough to cause immunosuppression which may have masked more subtle changes related to the immunological effects of blood.

Judson et al (1985) studied the effects of allogeneic blood transfusion before and after inoculation with a mammary carcinoma

cell line. There was no effect on survival if blood was given before tumour inoculation, but if the mice were transfused both before and after tumour inoculation, then this led to a prolonged survival.

Transfused syngeneic blood did not affect growth or number of azoxymethane induced colonic tumours in rats compared with a saline transfused control (Carty et al, 1989).

In an extensive series of experiments involving a range of transplantable and autochthonous tumours (fibrosarcoma, ependymoma, neurinoma, adenocarcinoma and lymphoma), Lehhard et al could not demonstrate any effects of allogeneic and syngeneic transfusion on tumour growth compared with control rats which were transfused with saline (Lehhard et al, 1989). They had demonstrated, however, that their transfusion regimes were associated with increased suppressor cell activity.

In 1981, Gantt had noted the beneficial effect of blood transfusion on renal graft prognosis (Gantt, 1981). He suggested that patients who are transfused may be immunosuppressed "to the point where the malignant tumour has a better chance to survive". He was aware that tumour antigens were similar to histocompatibility antigens and that the immunosuppression created by blood transfusions appeared to be non-selective for histocompatibility antigens.

Burrows and Tartter first drew attention to a possible detrimental effect of blood transfusion on survival and recurrence in colorectal cancer patients (Burrows, 1982). They reported a retrospective study of 122 patients who had undergone curative resections for primary colorectal cancer. Of these patients, 58 (48%) received blood transfusion. The cumulative recurrence-free survival rates were lower in the transfused patients. The transfusion effect was also present when left and right colonic lesions were considered separately. The conclusion was that perioperative blood transfusion was associated with recurrence after curative colon cancer surgery. They suggested that blood transfusion should be avoided if possible, and that if transfusion was essential then leukocyte-free blood should be given "to avoid induction of a tolerant state" citing Fuller (1982).

In response to this report, it was suggested that the observed difference in survival rates could be accounted for by difficulty of resection (Hodgson, 1982). This would be influenced by the location

of the tumour and by the skill and experience of the surgeon. The inference was that the tumours of patients requiring transfusion were more difficult to remove, and the operation involved more operative manipulation and bleeding than in the non-transfused group. This counter-argument has consistently been raised by critics of the hypothesis concerning the immunosuppressive effects of blood transfusion. However, subsequent retrospective studies have failed to address this criticism because there are no obvious prognostic factors which can identify a tumour which is difficult to resect.

Again in response to Burrows and Tartter's paper, Opelz reviewed the records of the Denver Kidney Transplant-Tumor Registry (Opelz, 1981). He found no evidence that non-specific immunosuppression by transfusions might lead to an increased risk of malignancies in kidney transplant recipients who had received blood transfusions compared with those who had not been transfused.

The next retrospective study to be reported involved 206 patients (Agarwal, 1983). Of these 65% were transfused within a month before or after surgery. The recurrence rate was 48% in the transfused patients and only 10% in those not transfused. For these two groups the mean follow-up was 39 months and 46 months respectively. The same authors (Agarwal and Blumberg) of this abstract subsequently reported a retrospective study in the British Medical Journal.

This study included the same patients, but the number analysed was now 197 (Blumberg, 1985). Recurrence or death due to recurrence was

observed in six of 68 (9%) patients not transfused. In the transfused group recurrence occurred in 56 of 126 patients (43%). These survival statistics were those at the end-point of the study. The five year recurrence-free survival was 90% and 50% approximately. No relationship was seen between the incidence of recurrence and the amount of blood transfused. This study is important because it was the first to use sophisticated statistical techniques to determine the relationship of transfusion rates with other variables. Using a multivariate stepwise logistic regression it was found that age, location of the tumour, duration of the operation and preoperative packed cell volume were the only factors tested which had a significant association with blood transfusion. Dukes' stage and year of operation were not associated. Tumour site was and of these (left, right and rectum) tumours in the rectum were associated with the highest incidence of transfusion while those in the left colon had the lowest association.

A prospective study was performed in 1981 in Auckland, New Zealand (Frankish, 1985). This included 179 patients who underwent potentially curative surgery for colorectal cancer. Of these 59% were transfused and this group included significantly more rectal tumours and patients undergoing operations longer than two and a half hours. At 42 months the recurrence-free survival was 73.6% for transfused versus 70.4% for non-transfused patients.

Another retrospective study used the Cox proportional hazards model to adjust the the two groups for differences in known prognostic variables (Nathanson, 1985). Between 1972 and 1977, 366 patients in

the Henry Ford Hospital, Detroit had curative resections for colorectal cancer. At 4000 days the transfused group had a survival rate of 43% compared with 56.5% in those not transfused. This was a significant difference when the logrank test was applied ( $p < 0.005$ ). However, after stage, age and sex had been adjusted for using Cox regression analysis, blood transfusion was not a significant prognostic variable. In fact the most significant prognostic variable in the study was the percentage of lymph nodes containing microscopic tumour deposits. They also found that patients with stage B2 tumours who were transfused had a better prognosis than non-transfused cases.

Foster et al found a 68% five year survival rate for non-transfused compared with 51% for transfused patients in 129 patients who had curative resection for colorectal cancer (Foster, 1985). However, the logrank test failed to demonstrate a statistically significant difference between the two survival curves.

Another group had been studying the effects of haemodilution on coagulation of blood (Blair, 1985). They had demonstrated that haemodilution led to a fall in haemoglobin and hypercoagulation. They subsequently showed that blood transfusion had an anticoagulant effect. It had previously been shown that anticoagulation prevented experimental circulating malignant cells becoming metastases (Hilgard, 1976). Consequently, 301 patients at St Mark's Hospital were studied. They reported that blood transfusion had no effect on survival in patients having curative resection of Dukes' A and B tumours. However, there was an apparent beneficial effect of blood



transfusion on the survival of patients with Dukes' stage C tumours. The five year survival for transfused patients being 65% (43/66) compared with 44% (14/32) for non-transfused patients. A simple chi squared test applied to these small numbers gave a significant difference ( $p < 0.05$ ). The other important point about this study is that blood transfusion was defined as blood given during the operation. This probably excludes a large number of patients who may have been susceptible to an immunosuppressive effect of blood transfusion giving either before or after surgery.

The influence of tumour site on the transfusion effect was examined in 207 cases by separately analysing right, left and sigmoid colectomy operations (Ota, 1985). This study did not demonstrate a detrimental effect of blood transfusion on five and ten year survival. The five year survival was 77% ( $n=45$ ) for non-transfused and 72% ( $n=162$ ) for transfused patients. The ten year survival figures were 61% and 71% respectively. There was a trend which showed a lower survival rate as the amount of transfused blood increased, but this was not significant.

Workers in Newcastle reported a series of 517 patients undergoing curative surgery for colorectal cancer (Parrott, 1986). They compared the recurrence and mortality rates in transfused and non-transfused groups of patients. The two groups were evenly matched for the major prognostic factors (age, sex, Dukes' stage and histological differentiation). The transfused group contained significantly more rectal tumours. Blood transfusion was given to 72% of the patients. The transfused group had a mean operative



blood loss of 837 ml compared with 333 ml in the non-transfused group. Transfused patients had a significantly longer operation (2.37 versus 1.99 hours). The recurrence free-survival rates were 60% for the transfused group and 76% for the non-transfused group. The logrank test suggested that this difference was highly significant. A similar trend was established when rectal lesions were excluded from the analysis. In this group of patients the probability of recurrent disease at five years was 20% greater in the transfused group.

Francis, who was one of the first to demonstrate an experimental transfusion effect in a tumour model, reported a retrospective study of 87 patients (Francis, 1987). Of these 67% were transfused. The recurrence-free five year survival was 64% in transfused patients and 74% in patients not transfused. This was not a statistically significant difference using the logrank test. However, there was a significantly higher incidence of recurrence in those patients who received transfusion during surgery when compared with those who were transfused either before or after surgery. It was suggested that factors influencing the need for blood transfusion during operation had a greater effect on prognosis than blood transfusion as a separate prognostic variable.

In an attempt to eliminate the epidemiological heterogeneity of colorectal tumours Creasy et al studied retrospectively 68 patients with sigmoid tumours (Creasy, 1987). The sigmoid colon was chosen because of the high frequency of tumours at this site and because about half of the patients with sigmoid tumours were transfused

(33/68). The five year recurrence-free survival was 53% for non-transfused patients and 28% for transfused patients. When the Cox proportional hazards regression analysis was applied it was found that histological stage and blood transfusion status were the only significant co-variants. In fact blood transfusion status was the most sensitive prognostic indicator for recurrence. There was no association between increasing number of units transfused and increased risk of recurrence. However, the correspondence columns published brief contradictory results of another study of 99 patients with sigmoid cancer (Kiff and Kingston, 1987). This was part of a prospective series of 525 patients who underwent curative resection for colorectal cancer.

Bickel et al reported (briefly) a series of 188 colorectal cancer patients (Bickel, 1985). This study did not demonstrate a difference in survival statistics between transfused and non-transfused patients.

A series of 281 patients was studied by a Canadian group (Corman, 1986). Using Cox's regression analysis they found that the number of units of blood transfused had a strong influence on prognosis in patients with colorectal cancer, particularly colonic cancer. This blood transfusion effect was not observed when rectal tumours were studied separately. It was suggested that this was due to the small numbers of patients involved. The transfusion rate for the whole group was one of the highest with 84% of 236 patients being transfused. This group carried out further studies of rectal cancer which did not confirm these initial results (vide infra: Arnoux et

al, 1988).

The most comprehensive retrospective study comes from the Large Bowel Cancer Project which is a collaborative study involving 94 surgeons and 38 pathologists in 23 hospitals in the United Kingdom. In this project clinical data were collected prospectively, but the blood transfusion details had to be collected retrospectively. It appears that complete records were obtained in only a quarter of the total cases involved. Despite this, the series included 961 cases with complete data - the largest retrospective study to date (Cheslyn-Curtis et al, 1988, 1990). There was no survival advantage to patients in either group of transfused or non-transfused patients. Blood transfusion was more likely to be given for tumours in the rectum and rectosigmoid, Dukes' stage C and if there was some degree of tumour fixation. Of those patients who developed metastases, there were significant differences in the organs involved. Transfused patients tended to have a higher proportion of extra-hepatic metastases. This could be explained by the larger number of rectal tumours in the transfused groups. Tumours in this site may metastasise through the systemic venous circulation rather than entirely through the portal venous system.

Beynon et al studied 519 patients with colorectal cancer (Beynon, 1988). Recurrence was diagnosed in 214 patients; 179 of these patients had been transfused whilst 35 had not. The incidence of recurrence was significantly higher in transfused patients with an increased risk of both local and distant recurrence. The risk associated with transfusion was greatest if the patient had been

transfused during definitive surgery for colorectal cancer.

Arnoux et al, studied 198 patients with rectal cancer, 96% of whom were transfused (Arnoux et al, 1988). It was found that the number of units transfused perioperatively had a detrimental effect on survival, and this was independent of the other analysed variables. Their data also indicated that the timing of transfusions may be important since peroperative transfusion had a greater prognostic significance than other transfusions.

Stubbs et al, studied a group of 59 patients who had undergone curative surgery for colorectal cancer and who had been followed for five years with prospective data recording (Stubbs et al, 1988). The survival rate for transfused patients was 55% compared with 65% in non-transfused patients: this was not a significant difference, however.

It had been suggested from animal studies that blood loss (as opposed to transfusion) enhanced development of metastases (Singh et al, cited in van Lawick van Pabst et al, 1988), however, this group showed that perioperative blood loss did not have prognostic significance in colorectal cancer patients. This study confirmed that perioperative blood transfusion was associated with poorer survival rates.

Crowson et al, studied a group of 525 patients undergoing prospective follow-up after colorectal cancer surgery (Crowson et al, 1989). Perioperative blood transfusion had been given to 71% of

these patients, but this had no significant effect on outcome.

Tiska-Rudman and colleagues (Zagreb) reported a detrimental effect of blood transfusion on survival in 312 patients with colorectal cancer (Tiska-Rudman et al, 1988). Follow-up was for 5 years, but over a third of the patients were lost. In transfused patients 64% developed metastatic disease, whereas in non-transfused patients no patient developed recurrence. However, non-transfused patients had a significantly better prognosis in terms of staging.

Data were analysed from a prospective trial which compared two different resection techniques: three quarters of these patients had been transfused, but the 5-year survival was the same (52%) for both transfused and non-transfused (Vente et al, 1989). Wobbes' study was of 270 patients and showed a survival disadvantage associated with blood transfusion which was worse if two or more units of whole blood had been given (Wobbes et al, 1989). However, the transfused group in this study had a much higher proportion of rectal tumours and this could explain the observed differences. Onodera et al demonstrated that blood transfusion status was the strongest prognostic indicator equal with Dukes stage (Onodara et al, 1989).

A small study of patients undergoing abdominoperineal resection for rectal cancer suggested that the need for blood transfusion in these cases was a significant risk factor for postoperative complications (Halpern et al, 1989). Hermanek et al, using multivariate analysis, showed no significant effects on survival of blood transfusion, but did find that administration of fresh frozen plasma had a highly

significant association with poorer survival.

In a study of 520 patients with colorectal cancer, blood transfusion was significantly associated with an increased recurrence rate, but further analysis suggested that this could be explained by other variables such as emergency operations for obstruction and involvement of other structures with the primary tumour mass (Mecklin et al, 1989).

Table 4.1

Influence of blood transfusion on survival in colorectal cancer  
patients

Author	n	%BT	Year of censor	% Survival		p value
				BT	No BT	
Burrows, 1982	122	47	5	51*	84	<0.005
Blumberg, 1985	129	43	5	57*	91	<0.0001
Foster, 1985	146	45	5	51	68	N.S.
Nathanson, 1985	366	54	10	43	56	<0.005
Blair, 1985	98**	67	5	65	44	<0.05
Frankish, 1985	174	59	3.5	74*	70	N.S.
Ota, 1985	207	78	10	72	77	N.S.
Bickel, 1985	180	53	5			N.S.
Parrott, 1986	517	72	5	60	76	<0.005
Corman, 1986	281	84	20	40	60	<0.02
Francis, 1987	87	61	5	64*	74	<0.01
Voogt, 1987	113	76	5	48	74	<0.01
Creasy, 1987	68***	49	5	28	53	<0.01
Weiden, 1987	171	60	7	81*	73	N.S.
Cheslyn-Curtis, 1990	961	61	8			N.S.
Beynon, 1988	519	84	5			<0.001*
Arnoux, 1988	198	96 decreased survival with more blood				

(continued on next page)

Table 4.1 (continued)

Influence of blood transfusion on survival in colorectal cancer patients

Author	n	%BT	Year of censor	% Survival		p value
				BT	No BT	
Blumberg, 1986,88,89	197	65	5	65	97	<0.05
Windle, 1987	141		6.5			<0.02
Tiska-Rudman, 1988	312	80	5	64*	100	?
Stubbs, 1988	103	71	5	65	55	N.S.
van Lawick, 1988	164	71	5	80	68	<0.05
Crowson, 1989	525	71	5			N.S.
Vente, 1989	212	73	5	52	52	N.S.
Wobbes, 1989	270	68	5	57	72	<0.001
Onodera, 1989	592		5			<0.001
Klingler, 1989	282	81	5			N.S.
Hermanek, 1989	(?A)		5			N.S.
Mecklin, 1989	520	68	5	66	81*	<0.001

\* Recurrence-free survival

\*\* Dukes stage C only

\*\*\* Sigmoid colon tumours only

(?A) - abstract only and data not included



The various retrospective studies are summarised in Table 4.1. Several points deserve attention. Of these studies, it should be noted that Blair found no transfusion effect in Dukes A and B cases, but found that transfused patients with Dukes C tumours had a significantly better prognosis despite the small numbers of cases studied, although this study considered transfusions given only during the operation. They postulated that blood transfusion had an anticoagulant effect and this mechanism protected patients from developing metastases from tumour emboli. Their finding is supported by another study which demonstrates a possible beneficial effect associated with blood transfusion in patients with stage C2 colon cancer (Weiden et al, 1987).

Clearly, there is a marked variation in the proportion of patients receiving transfusion. The reason for this is unclear, but it probably reflects different transfusion policies rather than differences between the various groups of patients.



Further evidence of an immunosuppressive effect of blood transfusion comes from a prospective trial of adjuvant 5-fluorouracil and levamisole in colorectal cancer (Windle, 1987). Following curative surgery for colorectal cancer 141 patients had been randomised to receive a six month course of chemotherapy alone or combined with the immunomodulating drug levamisole. A third group acted as controls. This trial was curtailed at an early stage of patient recruitment because of evidence of a possible detrimental effect on survival associated with levamisole. However, they were able to apply a series of Cox proportional hazards models using various factors as co-variants. This demonstrated that receipt of blood immediately before, during or within three days of surgery had a significant detrimental effect on survival ( $p=0.0166$ ).

Another prospective study of colorectal cancer patients has been reported by Tartter et al (1986a). This showed a significant association of blood transfusion with postoperative infective complications. These infective complications included wound infections, pneumonia, urinary infections and intra-abdominal abscesses. Interestingly, patients with a preoperative haematocrit of less than 40% had a lower incidence of postoperative infections.

The same group also analysed prospectively the indications for perioperative transfusion in another series of 123 patients undergoing surgery for colorectal cancer (Tartter, 1986b). They decided that if patients had a haematocrit of greater than 36%, then they did not require transfusion. Of the total, 41% were transfused

and by their criteria 28% of these patients received at least one unit of unnecessary blood. They concluded that overtransfusion resulted from excessive intraoperative transfusion and secondly it was due to the apparent practice of giving units of blood in pairs. They suggested that a greater use of haematocrit testing would reduce the amount of blood transfused unnecessarily. However, the major criticism of this proposal is that the haematocrit does not accurately reflect blood volume particularly during haemorrhage.

Further studies by Tartter investigated the relationship of blood transfusion to malignancy in 354 patients with colorectal cancer matched by age and sex to a control group of patients who were undergoing hernia repair or cholecystectomy (Tartter, 1988).

The incidence of previous transfusion was higher in the control group (17% versus 9%). Of the cancer patients, previously transfused patients and non-transfused patients had similar preoperative clinical features.

The amount of perioperative blood transfused was found to be an independent prognostic variable in a study consisting of 55 patients who had hepatic resections for colorectal liver metastases (Stephenson et al, 1988). It was calculated that for each additional unit of blood transfused the risk of disease recurrence and death was increased by 5% and 7% respectively.

There has been one prospective study of pre- and post- transfusion immune status (Quintiliani et al, 1988). The study group included a total of 38 patients undergoing colorectal cancer surgery of whom 20

#### 4.5. BLOOD TRANSFUSION AND NON-COLORECTAL CANCER IN HUMANS

The possible detrimental effects of blood transfusion are not as obvious in other malignancies. Several have been studied including breast, lung, gastric, renal cancer and sarcoma.

##### 4.5.1. Breast

Nowak reported a retrospective study of 81 patients who had surgery for breast cancer (Nowak, 1984). There was no significant difference in recurrence-free survival rates between those who received blood transfusions and those who did not. Subgroups based on age, nodal status, oestrogen receptor status and progesterone receptor status were analysed, but no detrimental effect was found to be associated with blood transfusion. Patients who were being treated with tamoxifen did have a reduced recurrence-free survival if they had been transfused. Of the 81 patients 50% were transfused. In the whole group the 5 year survival was 70% for the transfused group and 61.5% for the non-transfused group. Fifty patients received tamoxifen and of these 21 were transfused. The five year recurrence-free survival in this group (n=21) was 48% for transfused patients and was 80% for those not transfused. The Cox regression method gave a p value of 0.03 for the observed difference despite the small numbers involved.

Block et al reported a small series of T1T2, node negative breast cancer and showed that metastatic disease developed in 21% of

were transfused. Age-matched controls numbered 24. They tested the following parameters:

- peripheral blood lymphocyte counts
- T and B cell counts
- CD4 and CD8 numbers
- proliferative response of lymphocytes to  
phytohaemagglutinin
- serum IgG levels
- interleukin-2 and interferon gamma synthesis by  
peripheral blood lymphocytes
- Ig synthesis in culture by stimulated lymphocytes

There was a significant increase in IL-2 and INF-gamma production in culture supernatants of the stimulated lymphocytes and also increased immunoglobulin synthesis in cancer patients whether or not they had been transfused. No significant differences in these immune function tests were found between transfused and non-transfused patients either before or after surgery.

Hoh et al carried out a prospective study of immune function in patients undergoing surgery for colorectal cancer (Hoh et al, 1990). They measured the mitogenic activity of patient plasma using cultured fibroblasts as target cells. Transfused patients had a 100% increase over preoperative levels. Banked blood plasma was also tested in this experimental in vitro system. Mitogenic activity increased with storage time over 28 days, with the greatest changes occurring from the end of the second week.

transfused and in 21% of non-transfused patients (Block et al, 1984).

Foster et al studied 226 patients with breast cancer who had a mastectomy (Foster, 1984). Of these, 29% were transfused. When compared with the non-transfused group, both groups were matched for age, clinical stage and number of lymph nodes bearing metastases. Interestingly, patients who were not transfused had slightly larger tumours as measured by the pathologist. At five years for patients who had transfusions and those who did not, both the percentage of all deaths (22% and 21%, respectively) and deaths due to cancer (18% and 16%, respectively) were similar. No effect was seen with increasing amounts of blood transfused.

Tartter and Burrows did find that perioperative blood transfusion had a prognostic significance in their group of 169 patients undergoing mastectomy (Tartter, 1984). The five year recurrence-free survival was 51% for patients receiving blood and 65% for those not transfused. Both groups were comparable in age, clinical stage and proportion of radical mastectomies performed. They noted that preoperative haemoglobin levels were lower and operative blood loss was higher in patients who were subsequently transfused. They concluded that perioperative blood transfusion may be a significant prognostic factor for patients undergoing mastectomy for operable breast cancer.

In early breast cancer treated by mastectomy, Bickel et al showed that in 69 patients survival was prolonged in non-transfused

patients (n=51) compared with those transfused (n=17). The median survival was 63 months in the former and 28 months in the latter. This was stated as being statistically significant. A larger group (n=112) with more advanced disease was studied. There was no significant difference in survival between transfused and non-transfused patients.

Voogt et al, having identified a detrimental effect in colonic cancer (vide supra), found no effect of blood transfusion on survival in 383 patients who had a modified radical mastectomy for breast cancer (Voogt, 1987). A relatively large proportion of these patients were transfused (70%). The ten year survival was 57% for transfused and 52% for non-transfused patients.

Eickhoff et al, compared transfused with non-transfused patients in group of 96 women who had mastectomy (Eickhoff et al, 1988). The overall survival rates and the recurrence-free survival rates were similar in both groups. Similarly, a study of 230 patients with breast cancer treated at the Mayo Clinic, Minnesota, concluded that transfusions at or before mastectomy were not associated with increased recurrence or reduced survival (Kieckbusch et al, 1989). Cohen et al, found no evidence of a detrimental effect in mastectomy patients (Cohen et al, 1986).



#### 4.5.2. Lung

The first report concerning blood transfusion and lung cancer came from Burlington, Vermont (Hyman, 1984). Patients undergoing potentially curative resection for stage I and stage II carcinoma of the lung were studied (n=105). Both transfused and non-transfused groups were matched for sex, age, stage and histological characteristics of the tumour. There was a significantly higher proportion of right sided lesions in the transfused group. There was a trend which suggested that transfused patients had an inferior prognosis, but this was not statistically significant. The survival rate at five years was 44% in the non-transfused group and 27% in the transfused patients. However, after corrections for uneven distribution of other prognostic factors using the Cox regression method the significance of the association was enhanced ( $p=0.03$ ).

In order to eliminate variation due to technique, Tartter and Burrows applied their research to a personal series of 165 consecutive patients undergoing resection for stage I lung cancer (Tartter, 1984). Transfused patients had lower recurrence-free rates at five years than non-transfused patients (62% versus 76%). A much larger series from Milan was reported (Pastorino, 1986). This analysed 285 cases of stage I lung cancer undergoing potentially curative surgery. At eight years the survival was 40% for transfused and 41% for non-transfused patients. The recurrence-free survival rates were also similar: 36% and 34%, respectively.



Pachman et al, found that blood transfusion was associated with a lower 3 year survival in 195 patients with lung cancer (Pachman et al, 1988). This difference (62.5% in non-transfused versus 40% in transfused) was highly significant when stratified for T and N stages.

Keller et al determined the effect of perioperative transfusion on recurrence-free survival in 352 patients who underwent pulmonary resection for non-small cell lung cancer, but found none (Keller et al, 1989).

Moore et al showed an adverse effect of blood transfusion on early survival in a group of 330 patients with non-small cell lung cancer (Moore et al, 1989).

#### 4.5.3. Sarcoma

One hundred and fifty-six patients with high grade soft tissue sarcomas of the extremities were analysed to assess the effect of blood transfusion on survival and tumour recurrence (Rosenberg, 1985). These patients had all been entered in prospective trials, but the analysis of blood transfusion was retrospective. The five year recurrence-free survival rate was 70% in non-transfused patients compared with 48% in transfused. This was highly significant. Also, a direct relationship was found between the number of transfusions and the decrease in disease-free and overall survival: the greater the amount of blood transfused the worse the

prognosis. A large number of known prognostic variables were included in the analysis including age, sex, race of the patient, histology, site, resection margins, size of the tumour, operation time, adjuvant therapy, anaesthesia and individual surgeon. Accounting for these factors a strong association remained between blood transfusion and poor prognosis.

#### 4.5.4. Kidney

One study has found an association between blood transfusion and decreased survival in renal adenocarcinoma (Mikulin, 1986). Exactly half of 88 patients who had nephrectomy for this tumour received blood transfusion. The five year survival rates were 39% for transfused patients and 77% for non-transfused patients. The transfused patients were older and had larger tumours than those who were not transfused. They also had a higher incidence of venous invasion and more distant metastases at the time of surgery. Patients with metastases and lymph node involvement were excluded and two groups of patients with similar tumour sizes were selected. Transfused patients still had a significantly reduced survival.

A second study of 80 patients with surgically curable renal cell carcinoma did not show a significant effect of blood transfusion on survival (Mayonda, 1986). However, initial analysis had suggested a detrimental effect. Subsequent analysis showed this to be due to a higher incidence of stage pT2 and pT3 tumours in the transfused group.

Moffat has reported two series. The first included 67 patients undergoing surgery for renal carcinoma and they reported a survival advantage in the non-transfused group (Moffat et al, 1985). However, the same group studied a larger series of 126 patients of whom 63% had been transfused during their stay in hospital (Moffat et al, 1987). Log rank analysis indicated a significant difference between the survival experience of the two groups, but the relationship of confounding variables such as age and sex were tested and then perioperative blood transfusion no longer appeared to have an adverse effect on survival.

#### 4.5.5. Gastric

Two retrospective studies have demonstrated a detrimental effect on survival associated with blood transfusion. The first included a group of 232 patients with gastric cancer undergoing curative resections for gastric cancer (Kaneda et al, 1987). The effect was strongest in patients with Stage I gastric cancer. The administration of adjuvant immunochemotherapy (not specified) was seen to decrease the detrimental effect of blood transfusion on survival.

Another study of similar size found that perioperative blood transfusion was a significant prognostic factor for recurrence and survival after curative surgery for gastric cancer (Demmel et al, 1988). The Cox regression model was used for the multivariant

analysis. Transfusions were given to 82% of the 209 patients studied. The other two independent risk factors were tumour stage and lymph node involvement.

A study of 1000 Japanese patients showed that transfused patients had poorer 5-year survival than non-transfused (57% versus 81%), but this transfusion was not found to be a significant factor when confounding variables had been stratified (Kampschoer et al, 1989).

#### 4.5.6. Prostate

Heal et al demonstrated a higher frequency of recurrence of prostatic cancer associated with perioperative blood transfusion, although survival rates were not significantly different when compared with non-transfused patients (Heal et al, 1988). Notably, the risk of recurrence was greater in patients who had received whole blood.

McClinton et al, found that transfusion of non-autologous blood had a significant detrimental effect on survival after prostatic resection (McClinton et al, 1990). The study consisted of 246 patients of whom 29% had been transfused. As many others have done, they used Cox's model of proportional hazards regression analysis which allowed them to control for a number of variables which may affect survival; of these variables only age and transfusion status were found to have significant effects on survival.

#### 4.5.7. Head and neck cancer

Blood transfusion was associated with a poorer prognosis in a group of patients undergoing surgery for head and neck cancer, principally squamous cell carcinoma (Johnson et al, 1987). The disease-free survival rate declined progressively in patients given increasing numbers of transfusions. Jackson's study of 100 patients with head and neck cancer also suggested that blood transfusion also had a detrimental effect on survival (Jackson et al, 1989).

#### 4.5.8. Other tumours

Blumberg et al augmented their colonic research by studying the blood transfusion effect in prostatic and cervical cancer (Blumberg 1985, 1986). The first study used a proportional hazards model to demonstrate a significant risk associated with blood transfusion. The latter paper reported that in patients with colonic, rectal, cervical and prostatic tumours there was an association between transfusion of any amount of whole blood or larger amounts of red blood cells at the time of surgery and later recurrence of the tumour. This association was confirmed by the use of the Cox

proportional hazards technique. More than three units of red cells had a significant association with a poorer prognosis. Recipients of one unit of whole blood had a significantly higher recurrence rate (45%) than recipients of a single unit of red cells (12%). Recipients of two units of whole blood also had a higher recurrence rate (52%) than those receiving two units of red cells (23%). The authors proposed that a prospective trial using washed red blood cells may be the most effective way of addressing the question of the blood transfusion effect.

It has been shown that transfused patients undergoing surgical treatment of recurrent carcinoma of the vulva had a poorer prognosis than the non-transfused patients (Dalrymple et al, 1986).

Lastly, there has been one report of a spontaneous regression of a melanoma in a patient following a possibly incompatible blood transfusion (Cole, 1976).

Table 4.2

Blood transfusion (BT) on survival in cancer (excluding colorectal cancer)

Author	Tumour	n	%BT	% 5yr survival		p value
				BT	No BT	
Nowak, 1984	Breast	81	50	70*	62	N.S.
Tartter, 1984	Breast	169	23	51*	65	<0.05
Foster, 1984	Breast	226	29	78	79	N.S.
Block, 1984	Breast	53	36	79	79	N.S.
Cohen, 1986	Breast	(?A)		lower		<0.05
Bickel, 1985	Breast	195	35			<0.01
Eickhoff, 1988	Breast	96	29	53	52	N.S.
Kieckbusch, 1989	Breast	229	49	lower		N.S.
Hyman, 1985	Lung	155	68	27	44	<0.05
Tartter, 1984	Lung	165	36	51	65	<0.005
Bickel, 1985	Lung	198	53			<0.05
Pastorino, 1986	Lung	283	55	41**	40	N.S.
Pachman, 1988	Lung	195	45	lower		<0.05
Keller, 1989	Lung	325	39	lower	(8y)	N.S.
Moore, 1989	Lung	169	51	lower	(4y)	0.007
Rosenberg, 1985	Sarcoma	156	32	63	85	<0.0005

Table 4.2 continued

Blood transfusion (BT) on survival in cancer (excluding colorectal cancer)

Author	Tumour	n	% 5yr survival			p value
			%BT	BT	No BT	
Moffat, 1985	Renal	67	58	48	58	N.S.
Mickulin, 1986	Renal	88	50	39	77	<0.001
Manyonda, 1986	Renal	80	69	36	53	N.S.
Moffat, 1987	Renal	126	63	47	55	<0.05
Kaneda, 1987	Gastric	231	52	lower		<0.002
Demmel, 1988	Gastric	209	82	lower		<0.002*
Kampschoer, 1989	Gastric	1000	37	19	39-47	N.S.*
Heal, 1988	Prostate	262	14	10	19	N.S.***
McClinton, 1990	Prostate	246	29	18	35	<0.0025
Johnston, 1987	Head/neck	179		59	73 (2y)	<0.05*
Jackson, 1989	Head/neck	100		29	69	<0.05
Blumberg, 1985	Cervix	130				N.S.
Dalrymple, 1986	Vulva	146	53	75	90	<0.05

\* Recurrence-free survival

\*\* 8 year survival

\*\*\* higher frequency of recurrence in transfused group



#### 4.6. BLOOD TRANSFUSION AND IMMUNOSUPPRESSION IN OTHER DISEASES

##### 4.6.1. Inflammatory bowel disease

Tartter et al (1986) studied 59 patients undergoing intestinal surgery because of inflammatory bowel disease, 34 had Crohn's disease and 25 had ulcerative colitis. Blood transfusion was given to 73% with a mean of 2.95 units of blood. They counted peripheral blood lymphocytes. Blood lymphocyte counts were depressed following surgery, but returned to normal levels in patients not receiving blood. In the transfused patients blood lymphocytes remained depressed for six to eighteen months following surgery. Both groups of patients had similar severity of disease, similar steroid therapy and age etc. However, they did note that preoperative weight loss was less in the transfused group and they also gained weight faster after surgery.

Williams and Hughes reported a retrospective study of 60 patients who underwent small bowel resection for Crohn's disease (Williams and Hughes, 1989a). Of these, 28 had been transfused with 1-8 units of blood and the rest were not transfused. Both groups had similar clinical features apart from preoperative haemoglobin, length of bowel resected and site of bowel resection. The transfused patients had a significantly lower recurrence rate (19% compared with 59%), despite the fact that their clinical features would normally suggest a higher risk of recurrence. In subsequent published correspondence it was argued that disease location affected the risk of recurrence,

and when terminal ileal disease was considered the recurrence rate in transfused patients was 21% compared with 30% in non-transfused: clearly a less impressive result (Park and Russell, 1988; 1989). They also briefly reported a series of 87 patients with inflammatory bowel disease and found that blood transfusion did not seem to alter disease course.

Furthermore, Fry et al reported their series of 79 patients treated for Crohn's disease by bowel resection (Fry et al, 1989; Peters, 1989). Recurrence developed in 22% of transfused patients and in 44% of non-transfused patients with a follow-up period of 3 years. These results were independent of disease site. A larger series of 174 patients was reviewed (Sutherland et al, 1989). In the 72 patients with small bowel resections, there was no recurrence advantage associated with blood transfusion.

Williams and Hughes replied citing the advantages of their study, stating that all patients had small bowel disease, all surgery was performed by a single surgeon during a period when resection policy did not change, and that the data had been collected prospectively with standardised follow-up (Williams and Hughes, 1989b).

#### 4.6.2. Rheumatoid arthritis

Many years ago it was thought that blood transfusion may be of value in initiating recovery from acute rheumatoid arthritis. Rose (1984) describes experience from the early 1950s and noted that blood

transfusion was frequently followed by a temporary remission associated with a fall in erythrocyte sedimentation rate over a period of weeks. This phenomenon was apparently ignored as attention was diverted to the therapeutic advances associated with gold.

#### 4.6.3. Abdominal trauma

Perioperative blood transfusions have been associated with an increased incidence of postoperative infectious complications following surgery for penetrating abdominal trauma (Nichols, 1984).

#### 4.6.4. Anaemia

Fehrmen and Ringden (1982) demonstrated that lymphocytes of non-uraemic patients given multiple blood transfusions due to chronic anaemia had low mixed lymphocyte culture activity and also low mitogenic responses to phytohaemagglutinin. The influence of blood transfusion on T lymphocyte subpopulations in anaemic patients is reviewed in detail later.

The effects of transfusion were studied by Waymack and colleagues working with Alexander in Cincinnati (Waymack et al, 1986). They used a burned rat model to simulate trauma. They assessed cell-mediated immunity by measuring ear swelling in response to painting skin with dinitrofluorobenzene. Neutrophil function was assessed by measuring the ability of macrophages to phagocytose and kill bacteria (*staphylococcus aureus*). The ability of the macrophage to migrate into the peritoneal cavity in response to a chemical peritonitis was assessed. By injecting a known dose of the same bacterium into the dermis and by measuring colony formation from samples of the skin excised 24 hours later, they were able to assess dermal bacterial resistance.

Animals transfused with allogeneic blood demonstrated a marked suppression of cell-mediated immunity as measured by ear swelling. Macrophage migration was also adversely affected by transfusion although neutrophil migration was not. Neutrophil function was not decreased by transfusion. This series of experiments clearly demonstrated that blood transfusion influences some aspects of the immune system following injury.

This group from Cincinnati also studied the influence of transfusion on sepsis rates in a rat burn model (Waymack et al, 1987). Rats were given a 25% burn and this area of skin was challenged bacterially by painting on it a known quantity of *Pseudomonas*

aeruginosa bacteria. Allogeneic transfusion increased mortality unless it was given within 24 hours prior to the bacterial challenge when it diminished mortality. The authors offered two possible explanations for this. Firstly, allogeneic transfusion may cause an initial brief immunostimulation prior to the development of a longer-lasting immunosuppression. Secondly, it is possible that the transfused blood may have contained high levels of anti-pseudomonas antibody which were not present in the syngeneic blood used in the experiments.

...of the same source of ... and ...  
...play a ... role in the ... response ...  
...Prostaglandin E and other ... of ...  
...are now known to influence the immune system.  
...is perhaps the most important and this depends on ...  
...immunosuppressive effects.

#### Regulation of the immune response by macrophages

The macrophage plays an important role in the regulation of the immune response. This has been demonstrated both in vivo and in cell culture. The macrophage may have a non-specific ability to improve the viability of lymphocytes, or alternatively, the macrophage may suppress the proliferation of lymphocytes through secretion of a variety of substances. These include thyroxine, arginase, complement cleavage products, prostaglandin E and interferon. The macrophage also produces interleukin 1 which is one

#### 4.8. MACROPHAGE PROSTAGLANDINS AND IMMUNOSUPPRESSION

##### 4.8.1. Introduction

The macrophage is now known to have a key role in the regulation of cell-mediated immunity. Secondly, it has direct antitumour activity. Some of these effects are mediated by arachidonic acid metabolites secreted by the macrophage. Amongst white blood cells, the macrophage is the main source of prostaglandin synthesis and this cell may play a central role in the immunosuppressive response to blood transfusion. Prostaglandin E and other metabolites of arachidonic acid are now known to influence the immune system. Prostaglandin E is perhaps the most important and this compound has various immunosuppressive effects.

##### 4.8.2. Regulation of the immune response by macrophages

The macrophage plays an important role in the regulation of the immune response. This has been demonstrated both in vivo and in cell culture. The macrophage may have a non-specific ability to improve the viability of lymphocytes, or alternatively, the macrophage may suppress the proliferation of lymphocytes through secretion of a variety of substances. These include thymidine, arginase, complement cleavage products, prostaglandin E and interferon. The macrophage also produces interleukin 1 which is one

of the signals required for T cell proliferation. This latter response would appear to be associated with the antigen-presenting role of the macrophage. This involves the processing and presentation of antigenic molecules to the lymphocyte. This function is dependent on the compatibility of products of the genes in the major histocompatibility complex, namely Class Ia antigens. This interaction results in production of T cell lymphokines which activate the macrophage. The activated macrophage is able to phagocytose more easily and it secretes a variety of substances at a greater rate. These substances include neutral proteinases, interleukin 1, complement, mitogens, arachidonic acid metabolites, and hydrogen peroxide.

The macrophages are important in tumour immunity and they appear to have two major roles. Firstly, they act as antigen-presenting cells and secondly they are potential effector cells mediating tumour lysis. Macrophages require activation to become cytolytic to tumour cells. T cells secrete macrophage activating factor (MAF) which is a lymphokine following antigen-specific stimulation. It is likely that the macrophages depend on T lymphocytes for their role in the anti-tumour response. The true identity of MAF has yet to be clarified, but several lymphokines have been implicated. However, interferon gamma is known to render macrophages tumoricidal.

#### 4.8.3. Prostaglandins and immunoregulation

Most of the work on the role of arachidonic acid metabolites in the immune response has concentrated on the immunoregulatory role of prostaglandin E. In normal individuals the importance of prostaglandin E regulation is probably minor. However, in certain disease states, including cancer, which are associated with impaired cell-mediated immunity, it is likely that the prostaglandin E regulatory system is responsible for the immunosuppression. Of great importance is our ability to inhibit this immunoregulatory system with non-steroidal anti-inflammatory agents of which indomethacin is a good example.

#### Prostaglandin E<sub>2</sub>

This compound is produced in every tissue with the exception of mature red blood cells. It has a short half-life which limits its action to the site of its production.

The prostaglandins and the closely associated leukotrienes are groups of oxygenated fatty acids which have been detected in almost every tissue in man. Prostaglandins (and leukotrienes) are not stored in cells, but are formed by specific enzyme activity immediately before their release. Fatty acids are released from esterified lipid sources held within the cell membrane. The mechanism of release of these precursors is still unclear, but it is thought that they originate from the phospholipid reserves in the cell membranes. Phospholipase A is an important enzyme in this



release mechanism, and recent studies with platelets have shown that another enzyme, phosphatidylinositol-specific phospholipase C, will also release diacylglycerides, from which various lipases can form arachidonic acid (Rittenhouse-Simmons, 1979).

Currently, there are three known substrate fatty acids which are prostaglandin/leukotriene precursors. These are cis-5,8,11,14-eicosatetraenoic acid (arachidonic acid), and two related compounds, cis-8,11,14-eicoatrienoic acid, and cis-5,8,11,14,17-eicosapentanoic acid. Of these arachidonic acid is thought to be the most important hence the term often used to describe the biosynthetic pathway of prostaglandin - arachidonic acid cascade. Arachidonic acid is metabolized enzymatically via a group of lipoxygenase enzymes or by cyclooxygenase. Non-enzymatic oxygenation can also occur by ultraviolet light and by O<sub>2</sub>-generating systems. Enzymatic oxidation via cyclooxygenase results in the formation of a series of products, which include the prostaglandins. Leukotrienes are formed via the lipoxygenase pathways. Non-enzymatic oxidation of arachidonic acid generates chemotactic lipids (Ninneman, 1988).

Prostaglandin synthesis is carried out in steps by two membrane-bound enzymes, cyclooxygenase and endoperoxide isomerase, and by the soluble enzyme peroxidase (Figure 4.1). After membrane phospholipids such as phosphatidylinositol are released from their esterified state via phospholipase to form the stable prostaglandin precursor arachidonic acid, molecular oxygen is added by the enzyme cyclooxygenase to form prostaglandin G<sub>2</sub> (PGG<sub>2</sub>). PGG<sub>2</sub> is quickly

converted to PGH2 by the enzyme peroxidase. PGG2 and PGH2 are unstable , biologically active molecules which are intermediate in the transformation of arachidonic acid to prostaglandins. PGG2 and PGH2 have a half-life of about 5 minutes. Endoperoxide isomerase and peroxidase convert PGH2 to prostaglandin F2alpha, prostaglandin E2, prostaglandin I2 (prostacyclin). Prostacyclin is unstable and converts to 6-keto-prostaglandin F1 alpha. PGG2 and PGH2 are also the precursors of thromboxane A2. Thromboxane A2 is a potent cellular regulatory agent with strong platelet activating activity. Thromboxane A2 is rapidly hydrolyzed to thromboxane B2 which is inactive.

The bioactive prostaglandins are formed rapidly from their precursors, but are then quickly converted to metabolites with much weaker activities and these metabolites often have prostaglandin inhibitory activities. In the blood stream, PGE2 has a half-life of about 8 minutes (Hamberg et al, 1971) Sequential action of two enzymes, prostaglandin-15-hydroxydehydrogenase and prostaglandin-13-ketoreductase produce these 15-keto-13,14 dihydroxyprostaglandins which are metabolized further to urinary metabolites 7alpha-hydroxy-5,11,diketotetranorpropane.

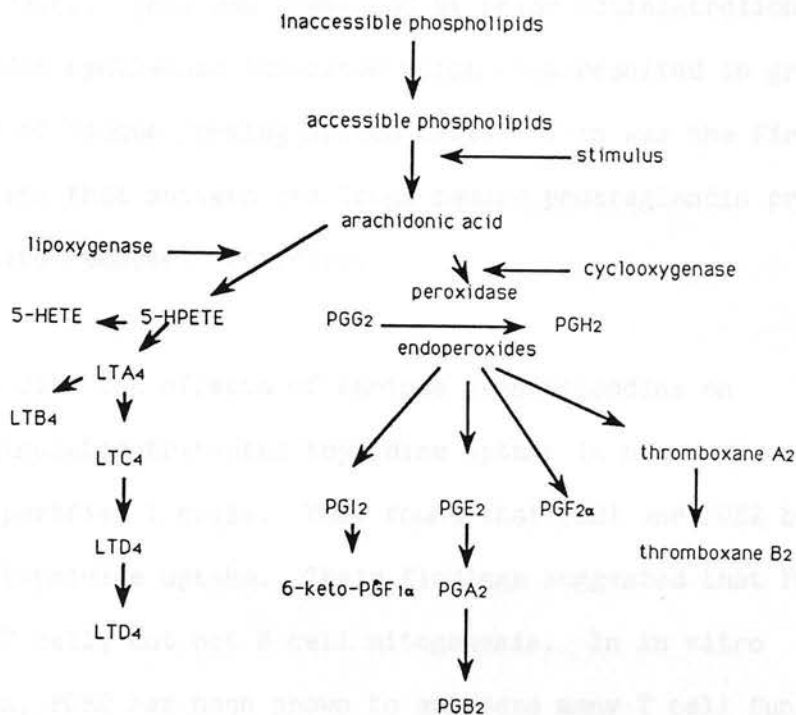


Figure 4.1

Major metabolites in the arachidonic acid pathways.

Antigenic challenge in mice by injection sheep red blood cells led to an increase in splenic production of prostaglandin (Webb and Osheroff, 1976). This was prevented by prior administration of a prostaglandin synthetase inhibitor which also resulted in greater production of plaque forming spleen cells. This was the first demonstration that antigen challenge caused prostaglandin production as a negative feedback mechanism.

Goodwin studied the effects of various prostaglandins on mitogen-stimulated tritiated thymidine uptake in human mononuclear cells and purified T cells. They found that PGE1 and PGE2 both inhibited thymidine uptake. Their findings suggested that PGE inhibited T cell, but not B cell mitogenesis. In in vitro experiments, PGE2 has been shown to suppress many T cell functions. These include mitogen responsiveness, clonal proliferation, antigenic stimulation, lymphokine production, the generation of cytotoxic cells in mixed lymphocytic cultures and lymphocyte migration. These studies have been reviewed (Goodwin and Ceuppens, 1983). In blood, the cell responsible for PGE2 production is a monocyte. Monocytes exposed to various antigenic stimuli have been shown to increase their production of PGE2 (Scott et al, 1982; Humes et al, 1982). This probably acts as a negative feedback mechanism to control the magnitude of the immune response.

One of the effects of this negative signal through release of prostaglandin E2 is the induction of suppressor T lymphocytes which inhibit interleukin 2 production in other T cells in the effector

arm of the immune response.

The ability of macrophages to activate suppressor T lymphocytes via a prostaglandin-mediated pathway was initially demonstrated by Webb and Nowowiejski (1981) in a series of experiments in which splenic macrophages were cultured and their ability to induce suppressor activity in T lymphocytes was assessed by measuring incorporation of tritiated thymidine after stimulation with phytohaemagglutinin. The suppression induced by macrophages was reversible by the addition of indomethacin.

Chouaib et al (1984) were able to induce T suppressor cells in culture by incubation with prostaglandin E2. They also examined the OKT4 (CD4) and OKT8 (CD8) phenotypes of the suppressor cells before and after induction. They found that the precursors were found mainly in the CD8 subset, but with some cells in the CD4 subset. However, induced suppressor cells were found in the CD8 subset only.

It was also suggested that the monocytes inhibit the interleukin 2 producing T cells by direct cell to cell contact.

Goodwin also cites studies which indicate that lymphocytes from healthy individuals over the age of 70 years are much more sensitive to inhibition by PGE2 than peripheral blood monocytes from young adults (Goodwin, 1983). Goodwin studied the effects of surgery and demonstrated a similar increased sensitivity of lymphocytes to PGE2 (Goodwin et al, 1981).

#### 4.8.4. Prostaglandins and natural killer cells

Natural killer cells are a heterogeneous collection of cells some of which may be related to the monocyte line, but others have characteristics in common with T lymphocytes. Natural killer cells are so called because of their capacity to lyse tumour cells without being previously sensitised.

There is evidence to suggest that prostaglandin E can inhibit natural killer cell activity in experimental models. Voth et al tested the effect of indomethacin on natural killer cell activity in a peritoneal macrophage model (Voth et al, 1986). Indomethacin caused a marked activation of natural killer cell activity against tumour cells. This activation was reversed by adding prostaglandin E<sub>2</sub>. They also demonstrated a second cell type with killer activity after administration of indomethacin and these cells were thought to have been macrophages. Prostaglandins have been shown to inhibit natural killer cell activity in mice (Brunda, 1980).

#### 4.8.5. Prostaglandins, prostaglandin synthetase inhibitors and immune regulation in cancer

Prostaglandins have been shown to enhance carcinogenesis in a mouse skin tumour model (Lupulescu, 1978). In tumour bearing mice, Pelus and Bockman (1979) demonstrated an increased prostaglandin synthesis by macrophages.

In colon cancer patients, prostaglandin mediated immune suppression of peripheral blood T lymphocyte mitogenesis is increased and this abnormality can be partially reversed by indomethacin added to the cell cultures (Balch et al, 1984). This study found that the depression of the immune response was greater in elderly patients and in patients with metastatic disease. Production of various arachidonic acid metabolites by peripheral blood monocytes was measured in this study. It was found that only PGE2 production was raised.

Similar studies have also demonstrated that indomethacin enhances the mitogen response of T lymphocytes in patients with melanoma (Tilden et al, 1981), head and neck cancer (Balch et al, 1982), Hodgkin's disease (Goodwin et al, 1977), and lung cancer (Han and Takita, 1980).

#### 4.8.6. Prostaglandins and immune regulation in burns

Patients with severe burns are known to be immunosuppressed due to the direct effects of the burn or because of the general immunosuppressive effects of major trauma. They are also a group of patients which is likely to receive multiple blood transfusions. The major manifestation of this immunosuppression is sepsis. Therefore cell-mediated immunity in burns victims has been well investigated.

It is known that burns patients have a decreased ability to produce

the T lymphocyte growth factor interleukin 2 and this finding was associated with suppression in the number of T-helper (CD4) lymphocytes which persisted for a long time during the recovery period (Wood et al, 1984).

This group also described similar findings in patients with non-burn injuries. They followed this research by establishing an experimental model of thermal injury. Mice were given a 25% scald and the splenocyte production of interleukin 2 was measured. Normally it is suppressed in these mice, but after administration of indomethacin to the culture medium, interleukin 2 production was enhanced (Wood et al, 1987).

In a separate series of experiments, the effects of exogenous prostaglandin E2 on lymphocyte blastogenesis and interleukin 2 were studied. It was found that thermal injury was associated with an increased susceptibility to the inhibitory effects of prostaglandin E2. This work clearly suggests a prostaglandin E2 action is involved with this reduction of interleukin 2 production.



#### 4.9. PROSTAGLANDINS AND BLOOD TRANSFUSION

##### 4.9.1. Dialysis patients

It is known that patients on chronic haemodialysis who receive multiple transfusions have comparatively high levels of prostaglandin E in their blood (Lenhard et al, 1985; Jackson et al, 1985; Roy et al 1985).

##### 4.9.2. Transplant models

Administration of prostaglandin has been shown to prolong the survival of murine skin allografts while prostaglandin synthetase inhibitors decrease graft survival (Anderson et al, 1977).

It has been shown that indomethacin blocks the blood transfusion induced immunosuppression associated with a prolonged graft survival in a rat heart transplant model (Shelby et al, 1987). They also demonstrated that anti-PGE antibody blocked allogeneic blood induced suppression by neutralising endogenous PGE in the experimental animals.

##### 4.9.3. Blood transfusion models

Waymack (et al, 1987) investigated the effect of transfusions on the production of arachidonic acid metabolites by cultured macrophages.

Using a rat model they demonstrated that allogeneic blood transfusions decreased macrophage migration in response to inflammatory stimuli. This response was elicited by injecting brain-heart infusate into the peritoneal cavity. Allogeneic blood transfusion was also associated with increased macrophage production of prostaglandin E2. Control groups given infusions of Ringer's lactate solution or syngeneic blood yielded more macrophages after peritoneal lavage and these harvested macrophages produced less prostaglandin E2 in culture.

#### 4.10.        INDOMETHACIN AND CANCER

Pollard et al (1977) demonstrated that treatment of tumour bearing rats with indomethacin retarded the rate and extent of metastases of prostatic adenocarcinoma cells. There is also evidence that prostaglandin inhibitors such as indomethacin and aspirin inhibit the growth of various transplanted tumours (Levine et al, 1972; Sykes and Maddox, 1972; Lynch et al, 1978; Lynch and Salomon, 1979).

Indomethacin has been shown to influence the course of colonic carcinogenesis. Pollard and Luckert (1980) induced colonic tumours in Sprague Dawley rats by administering five weekly doses of dimethylhydrazine by gavage. Treatment with indomethacin reduced the incidence of colonic tumours significantly. Also in the rats treated with indomethacin which did develop tumours, the tumours were smaller in number and size compared with the controls.

Indomethacin was administered by dissolving it in drinking water at a concentration of 20mg/L. Normal rats given a similar dose of indomethacin survived for 6 months with no evidence of clinical illness. However, autopsy on these rats showed that they had developed small knobs on the small intestine. Histologically they contained connective tissue, capillaries and polymorphonuclear leucocytes. Normal rats treated with a higher dose of indomethacin in the drinking water (40 mg/L) were found to be unwell after nine days and all had died by seven weeks. They had developed purulent peritonitis.

The same group also studied the effect of indomethacin on the development of colonic tumours in rats given a single injection of the acetate derivative of dimethylnitrosamine (Pollard and Luckert, 1981a, 1981b). It is known that this carcinogen is inactivated within 48 hours of injection into rats. In this experiment indomethacin therapy was commenced 14 days after the single injection of carcinogen. It is unlikely, therefore, that the mechanism involves the initial carcinogenic action of the chemical. A further series of experiments demonstrated a prolonged antitumour effect of indomethacin on chemically induced colonic tumours (Pollard and Luckert, 1983). In these experiments indomethacin was introduced to the rats as late as 66 and 77 days after the carcinogen had been administered, and the antitumour effect was demonstrable for at least 40 weeks.

Caignard et al (1984) reported results which conflicted with those of Pollard. They used Sprague Dawley rats and induced colonic tumours by giving five doses of dimethylhydrazine. There was no significant difference between the controls and rats treated with indomethacin (4/13 and 5/14 rats developed colonic tumours respectively). They also studied the effect of indomethacin on the development of transplanted colonic tumour cells and found that there was none. The same group also found that indomethacin treatment did not modify the growth of tumour produced in rats by injection with syngeneic colon cancer cells (Olsson et al, 1984).

The induction of colonic tumours by dimethylhydrazine was abrogated by synchronous treatment with indomethacin in a study of Sprague Dawley rats (Rubio et al, 1989).

## Summary

Macrophages activated by antigenic stimulation can induce T lymphocytes which have suppressor activity. This appears in part to be mediated by an increase in macrophage synthesis of prostaglandin E2. This can be reversed by prostaglandin synthetase inhibitors such as indomethacin.

There is also evidence that the immunosuppressive effects of blood transfusion are associated with increased synthesis of prostaglandins by macrophages. Similarly, indomethacin may reduce prostaglandin synthesis by macrophages following allogeneic blood transfusion and so reverse the immunosuppressive effect of blood transfusion, assuming that the effect is due to PGE2 and not another cytokine.

There is experimental evidence which demonstrates that indomethacin itself can influence tumour behaviour and cause inhibition of tumour growth. However, colonic cancer models have yielded conflicting results in this respect.

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## 5.1. CLINICAL RETROSPECTIVE STUDY

### 5.1.1. Patients

Case records were retrieved for all patients (314) who underwent laparotomy for colorectal cancer during the four year period 1977 to 1980 in Derbyshire Royal Infirmary and Derby City Hospital.

Identification of cases was performed in three ways:

- a) The theatre operation records were complete for this period and all operations performed at both hospitals were recorded in the operation books. The initial data base consisted of names, hospital numbers and dates of all operations of the following categories: bowel resection of any type, undefined laparotomy, specific colorectal cancer operations.
- b) Patient details were retrieved from the Department of Pathology records on all specimens categorised as colonic or rectal cancer.
- c) During the period of the study, the majority of patients had been entered in the Large Bowel Cancer Project. A complete listing of these patients was obtained from the Large Bowel Cancer Project database.



Case records were then obtained from the hospital Medical Records Department. It had been intended to extend the study back to include the years 1974 and 1975, but the cases notes of patients who had died during this period had recently been destroyed without microfilm recording (due to economic constraints).

The cases notes were then searched to obtain the relevant clinical details. Blood transfusion status was ascertained from the case notes. All patients cross-matched by the blood bank in hospital had a form inserted into their case notes. This form recorded details of blood group, amount of blood cross-matched and the number of units used, together with the relevant dates. Transfusion procedures at ward level required a thorough checking process to ensure patients received the correct cross-matched units. This system was the current safeguard against mis-matched transfusions and was adhered to rigidly by medical and nursing staff. The person checking the blood pack had to write the pack number and time on the transfusion form in the case notes. Therefore, the presence of a transfusion form yielded an accurate record of the amount of blood transfused and the date of transfusion. The absence of a transfusion form implied that the form had been lost or that the patient had never been cross-matched.

The hospital Blood Bank held records of blood issue and blood returns, but these records could not confirm that a particular patient had received the unit(s) not returned to the Blood Bank. Transfusion forms were found in the case notes of 304 patients, therefore complete transfusion data were not obtainable in 10

patients. It would have been possible for a patient to have had a transfusion form which indicated that no units were used during the perioperative period, but who subsequently was cross-matched again and transfused with the second transfusion form being lost. It is felt that the chances of this happening were small. To exclude this possibility, the Blood Bank records should have been checked against each patient in the colorectal cancer database. However, the lack of computerization of the Blood Bank records made this task impracticable, as the Blood Bank served a wide area of specialities, including two large District General Hospitals, a Children's hospital and several other smaller units. Pathological information was also obtainable from duplicate records kept in the Pathology Department.

Complete data sets (including clinical details) were obtained for 302 patients. Of these patients, 25 died within 30 days of surgery. These perioperative deaths were due mainly to cardiovascular and respiratory complications. A further 41 patients had palliative procedures for unresectable tumour or were found to have metastatic disease. Thus, 236 patients had curative surgery.

In order to limit the analysis to patients who had an abdominal operation only, the 77 patients who had abdomino-perineal resections were excluded from the second survivorship study which, therefore, comprised 159 cases.

### 5.1.2. Definitions

Curative resection was defined as the removal of the primary tumour which was histologically complete, with no evidence of distant metastases at the time of surgery. Perioperative blood transfusion means transfusion of whole blood or packed cells received during the period from initial presentation until 30 days following tumour resection. Some patients were found to have had transfusions after this point for a variety of reasons (e.g. prostatic surgery), but these were disregarded because of the unreliability of this information for all patients in the study who may have been treated at other centres.

### 5.1.3. Follow-up data collection

Follow-up data were derived from the case records, but supplemented where necessary from the Derby Cancer Registry and the collaborative study of colorectal carcinoma called the Large Bowel Cancer Project (Phillips et al, 1984). Given the resources available at the time it was not possible to trace patients who were lost to follow-up and not confirmed to have died, by contacting the appropriate general practitioner.

#### 5.1.4. Computers and statistical programs

The data for the retrospective study were entered into a statistical system called Minitab (Ryan et al, 1985). The life table analysis data were processed using a BBC "B" microcomputer. Initially, a statistical program called BSTAT was used (Feltham, 1985).

#### 5.1.5. Statistics

The group of patients who received perioperative blood transfusion was compared with the group of patients who had not been transfused with blood. Clinical details, known prognostic factors and survival data were assessed and compared using a variety of statistical methods:

##### a) Student's t test

The unpaired t test was calculated using the following formula:

$$t = D / \text{sqr} (SV (N1 * N2) / (N1 + N2))$$

where t = value of t

D = mean difference between groups

SV = pooled variance

N1 = number of subjects in group 1

N2 = number of subjects in group 2

b) Chi square test

the chi square statistic is

$$\chi^2 = \sum (\text{observed} - \text{expected})^2 / \text{expected}$$

c) Life table analysis

The method used to construct the life tables in the clinical studies was that described by Armitage (1971).

d) Mantel-Haenszel method

In the clinical study, this method was used to compare the survival and recurrence-free survival curves of the group of transfused patients with those of the group of non-transfused patients. This method was described by Mantel and Haenszel (1959) and the application of the method is further described by Schwartz et al (1980).

e) Logrank test

The logrank test is an approximate form of the Mantel-Haenszel method (Peto et al, 1977). It uses a chi square test for comparing the observed and expected numbers of deaths (or recurrence) in two groups. The relative simplicity of this test has led to its frequent use in clinical studies. It is known that the value of chi squared will always be less than the square of the normal deviate given by the Mantel-Haenszel method (Schwartz et al, 1980).

## 5.2 COLON CARCINOGENESIS MODEL

### 5.2.1 Introduction

A number of chemical carcinogens have been discovered which induce colonic tumours in animals. When administered to rats and mice these compounds produce colonic tumours which are similar in many respects to human tumours. The ideal carcinogenic agent should be organ specific and not be toxic to other organs. In fact, most colonic carcinogens produce tumours in other organs.

The first experimental induction of intestinal tumours involved mice fed with dibenzathrene or methylcholanthrene (Lorenz and Stewart, 1941), but these tumours were found in the small intestine only and not the large bowel. It was then found the feeding some inbred strains of hamsters 3-methylcholanthrene caused males, but not females, to develop colonic tumours (Homburger, 1972). In the 1950's it was known that there was a high incidence of bladder carcinoma in the dye industry workers. One of the byproducts of the manufacture of dyes is the biphenyl group of compounds. Some of these compounds were found to produce small and large bowel tumours in rats (Walpole, 1952). A familial syndrome resembling motor neurone disease (amyotrophic lateral sclerosis) had a high incidence in Chamorro tribe in the island of Guam. It was thought that a dietary factor may be involved in the aetiology of this disease. The nuts of the tropical plant *Cycas circinalis* were ground to make cycad meal which was eaten by the tribe. Rats were used to study

the disease. However, rats fed with cycad meal did not develop the neurological pathology, but some of the animals were found to have adenocarcinomas of the colon (Laqueur et al, 1963). This group subsequently proved that the carcinogenic agent in cycad meal was cycasin, a glucoside of methylazoxymethanol. Cycasin did not prove to be a useful experimental carcinogenic agent because it was an unstable compound and cycad nuts were apparently hard to come by. In fact, cycasin was not carcinogenic when administered parenterally to rats. The carcinogenicity is due to splitting of the cycasin by bacterial glucosidase in the rat intestine. This derivative is methylazoxymethanol.

Working in Freiburg, Druckrey studied a series of compounds related to methylazoxymethanol (Druckrey, 1970). The main chemicals used were azoxymethane and its precursors, azomethane and 1,2-dimethylhydrazine. These chemicals are now known to be the most effective carcinogens for selective induction of intestinal tumours in rats, and also in mice. Azoxymethane is thought by some to be the most effective carcinogenic agent, but this chemical may increase the yield of small bowel neoplasms in rats (Nigro et al, 1973).

#### 5.2.2. Dimethylhydrazine

Roe reviewed the experience of nine manufacturers of hydrazine who had analysed the health of their workers (Roe, 1978). Five of these companies had manufactured hydrazine since years which ranged from 1940 to 1956. A total of 423 workers were involved and there was no



evidence to suggest an increased risk of death related to exposure to hydrazine or by-products.

The chemical dimethylhydrazine is unstable at room temperature and is rapidly dehydrogenated in the presence of trace metals. Therefore, the chelating agent ethylene-diaminetetracetate (EDTA) is added to the solution to prevent this (Haase, 1973).

#### 5.2.3. Dimethylhydrazine and the animal model

##### Metabolism of dimethyl hydrazine

Dimethylhydrazine is metabolised and conjugated in the liver and is then secreted in bile (LaMont and O'Gorman, 1978). The hepatic metabolism probably involves the cytochrome P-450 pathway (Hathway, 1984). The active carcinogen is methyl diazonium which is an alkylating agent. This metabolic activation does not require the presence of bacterial gut flora. The active agent reaches colonic mucosal cells by direct intestinal transit and also through the blood stream. The basic carcinogenic effect is known to be the methylation of DNA within the colonic mucosal cells. This methylation of DNA also occurs in the liver and kidney, but these organs are not the prime target of the carcinogenic process.

## Course of carcinogenesis

With carcinogenic treatment there is a generalised increase in cellular proliferation and mitotic figures can be seen in the upper third of the crypts and on the surface epithelium. Then there is a decrease in the number of goblet cells, hyperplasia of the glands and areas of focal atypia. These changes have been shown to occur after two or three months of treatment.

The first appearance of microscopic adenomas and adenomatous polyps is between four and six months after starting treatment. These polyps then grow and may undergo malignant change. They commonly cause rectal bleeding and may cause intestinal obstruction either by stenosis or intussusception. The disease process progresses with peritoneal metastases rather than involvement of other organs. However, hepatic metastases are not uncommon. Pulmonary metastases are uncommon. In Druckrey's work, the mean induction time for colonic tumours was 184 days using a weekly dose of 21 mg/kg of dimethylhydrazine. If a reduced dose was used (7 mg/kg) the tumour induction time increased to an average of 333 days. He also found that a lower dosage of 3 mg/kg given orally in the drinking water did not produce colonic tumours, but they did develop malignant haemangiomas of the liver.

## Rat colon anatomy

The normal anatomy of the rat colon and the histopathology of chemically induced colonic tumours were studied by Lindstrom (1978). The colon in the rat varies in length (21 to 27 cm) and five main segments are recognised: the caecum, proximal colon, major flexure, distal colon and rectum. The caecum and proximal colon are fairly mobile and are attached to a mesentery which is shared with the small bowel. The proximal colon when opened has a characteristic herring bone pattern on the mucosal aspect. The next segment, the major flexure, is short and the mucosal pattern consists of longitudinal folds. These folds continue into the distal colon and rectum. Plaques of lymphoid tissue are to be found throughout the length of the bowel. These lymphoid patches may be important in the pathogenesis of chemically induced carcinogenesis. A strong association has been found between this gut associated lymphoid tissue and adenocarcinoma of the colon (Deasy et al, 1982). It was reported that a definite lymphoid patch existed at each of the colonic sites where dimethylhydrazine-induced tumours occur, but not at sites where tumours were not found.

## Rat colon histology

Histologically, the rat colon and rectum consist of a lamina propria, muscularis mucosa, and an external layer of smooth muscle. This layer consists of an inner circular and an outer longitudinal

layer. There is also a subserosal layer under the serosa. The mucosa varies depending on the part of the colon. In the caecum the tubular glands are lined with columnar epithelium and goblet cells which tend to be evenly distributed among the various parts of the tubules. In the proximal colon the tubules are branched. The mucous membrane of the major flexure is thicker with longer tubular glands. The mucosa is thinner again in the distal colon and is similar in the rectum. Lymphoid plaques consist of collections of lymph follicles in the boundary between the mucosa and the submucosa and occasionally it is seen to break through into the muscularis mucosa. He also described a special type of goblet cell in the basal parts of the tubular crypts in the proximal colon of the rat. The staining properties of these cells demonstrated a faint or negative PAS reaction and a strong reaction with alcian blue, but negative for sulphomucins. It was proposed that this cell represented a third type of mucosal cell apart from the normal columnar epithelial cell and the normal goblet cell. This third cell type has no counterpart in human colonic mucosa.

#### Histopathology of dimethylhydrazine carcinogenesis

The histopathological features of dimethylhydrazine induced colonic tumours have also been studied by Ward (1974). Mucinous adenocarcinomas occur primarily as sessile or plaque-like lesions in the proximal half of the colon. Many occur adjacent to lymphoid follicles. These tumours consist mainly of mucus-laden signet ring cells. The serosa may be invaded and the tumours metastasise to the

peritoneum and the regional lymph nodes. Polypoid adenomas and nonmucinous adenocarcinomas occur more frequently in the distal half of the colon. Polypoid lesions can be classified as either polypoid adenocarcinomas or polypoid adenomas containing areas of extreme atypia or carcinoma-in-situ, but without invasion of the stalk. Rats also develop well-differentiated adenocarcinomas of the duodenal mucosa and also, less frequently, of the jejunal mucosa. Most of these tumours arise close to the orifice of the bile duct and it has been suggested that an activated carcinogen is secreted in the bile.

#### 5.2.4. Dimethylhydrazine Carcinogenesis Procedure

##### Preparation of reagents

The carcinogen was obtained from Aldrich Chemicals in the form of 1,2-dimethylhydrazine 2HCl. The chemical was processed in batches which consisted of 10 g of the anhydrous powder.

The dimethylhydrazine was prepared in a fume cupboard under aseptic conditions. During these procedures disposable rubber gloves and a plastic gown were worn. A two litre flask was placed on a magnetic stirrer and one litre of 0.9% saline was poured into the flask. To this was added 15 g of ethylene-diaminetetracetate (EDTA) (Sigma: Poole, UK). This was done to prevent dehydrogenation which occurs

rapidly in the presence of trace metals (Druckrey, 1970). The pH of the solution was brought to pH 7.0 using molar  $\text{NaH}_2\text{CO}_3$  (Sigma, Poole, UK). The pH was measured using a pH meter. Then 10 g of dimethylhydrazine was added gradually. Again the pH was corrected - in fact the dimethylhydrazine would not dissolve if the acidity was high. The volume of the batch was 1.08 l. After the compounds had been seen to have dissolved completely, the solution was sterilized by filtration through a 0.22  $\mu\text{m}$  filter unit (Flow Laboratories: Irvine, UK) divided into 20 ml aliquots which were placed in sterile universal containers (Sterilin: Teddington, UK). These were labelled and frozen at  $-70^\circ\text{C}$ . The aliquots were removed and allowed to return to room temperature prior to injection.

The initial dose used was 16 mg/kg. Therefore, a rat weighing 200 g received 0.35 ml, a rat weighing 500 g received 0.86 ml, and a rat weighing 750 g received 1.29 ml of the carcinogenic solution.

#### Control solution

A control solution was used in the initial experiments. This was prepared in a similar manner, but the dimethylhydrazine was omitted. This represented a 1.5% solution in saline of ethylenediaminetetracetate (EDTA) (Sigma: Poole, UK) with pH corrected to pH 7.0 by addition of  $\text{NaH}_2\text{CO}_3$  (Sigma: Poole, UK) in molar concentration. This was also divided into 20 ml aliquots and frozen as described above.

## Animals and method of administration

Male Sprague Dawley rats were bought in (A Tuck and Son Ltd, Raleigh, UK) and were admitted to the animal facilities weighing 150-200 g. The animals were fed on a standard pellet diet (Pilsbury's Modified) and tap water ad libitum. The rats were individually numbered using system of ear punching. After one to two weeks acclimatisation the rats were examined and any ill animals destroyed. The animals were given weekly injections of 16 mg/kg dimethylhydrazine, using sterile disposable hypodermic needles (Becton Dickinson: Wembley, UK) and sterile polypropylene syringes (Becton Dickinson: Wembley, UK). The animals were restrained manually without anaesthesia and were weighed prior to injection. The exact dose was calculated and the carcinogen was given by the subcutaneous route. Disposable gloves and plastic aprons were worn while handling the solution and during the injections. The total number of injections given to each rat was 16 initially, but was reduced to 14 in the last series of experiments.

## Assessment of carcinogenesis

The rats were killed at the appropriate time by ether anaesthesia. The animals were weighed and inspected for external signs of pathology. The most obvious signs were related to the development of tumours of the external auditory canal.

A midline incision was made in the abdominal wall. The presence of ascitic fluid was noted. The incision was extended into the thorax to allow removal of the lungs. The lungs were palpated and sliced at intervals with a scalpel in order to look for pulmonary metastatic deposits. The large and small intestines were brought out through the abdominal wound. The midline incision was extended downwards through the scrotum towards the anus. The muscle layers beneath this were incised to expose the pubic bones. The circumference of the anus was incised and the anus then lifted using toothed forceps. The anal canal and lower rectum could then be dissected to the floor of the pelvis. The testicular tissues were brushed aside. Next the bladder and seminal vesicles were held up in toothed forceps. These were removed by cutting across the base of the bladder with scissors. The anterior part of the pelvic ring could now be clearly seen. A blade of a pair of blunt-ended strong dissecting scissors was passed caudally beneath the pubic symphysis. This structure was then cut and the two ends of the pelvic ring separated by spreading the scissors. The pelvic rectum could now be seen encircled by the sphincters which were divided anteriorly. The anus was picked up in toothed forceps. This traction enabled the colon to be dissected from the posterior abdominal wall. As the mid portion of the colon was reached the small bowel and caecum were rotated to their embryonic position. The caecum and large bowel were then removed intact and placed in 0.9% saline (Sigma: Poole, UK).

A careful search was made of the visceral and parietal peritoneum for metastatic deposits. The greater omentum was spread and closely



examined as this was the commonest site of peritoneal tumour deposits. The small bowel was removed whole and placed in 0.9% saline. The liver was removed and weighed and sliced in sections to look for hepatic metastases. The spleen was inspected. Both kidneys were removed and examined for the presence of renal cysts.

The large bowel was opened longitudinally with scissors. The caecum was removed at this point to facilitate examination. Faecal material was washed off with fresh saline. The length of large bowel, which measured about 30 cm, was placed on a metal rule. The length was noted. The position and diameters of each macroscopic tumour were noted. Many tumours were related to the lymphoid patch found in the wall of the rectum and diagrams were made of these tumours. The diameters were measured using calipers. Magnification was obtained using a 1.5 binocular loupe. The large bowel was then wrapped like a swiss roll on a wooden rod and placed in 10% formalin (phosphate buffered) solution. The caecum was opened and the position and size of any tumours were noted. The small bowel was opened longitudinally and any tumours were noted. In the initial series of experiments the cranium was also opened and the brain removed. This was serial sectioned with a scalpel and a search made for possible tumour deposits. This was not a rewarding procedure and was later abandoned.

## Histopathology

Sections were taken from individual tumours arising in the rat bowel wall which had been fixed in formalin. These were processed automatically and embedded in wax. This procedure was performed by technical staff in the Department of Pathology, (Western General Hospital, Edinburgh) under the direction of Dr I Nawroz who was the Pathologist collaborating in this project. Sections were cut and mounted on glass slides and stained with haematoxylin and eosin.

The sections were then examined by the Pathologist who was unaware of the distribution of experimental groups. Details of the histopathological features were recorded. "Tumours" were classified as normal mucosa, adenoma or carcinoma according to established definitions (Morson and Sobin, 1976). Adenocarcinoma consists of columnar or cuboidal epithelium with varying degrees of loss of the normal differentiation pattern. The histological classification was based on the WHO definitions of grade: G1 is the most differentiated with well-formed tubules and the least nuclear polymorphism and fewest mitoses, G3 is the least differentiated, having only occasional glandular structures, pleomorphic cells and a high incidence of mitoses, while G2 is intermediate between G1 and G3 (Hermanek, 1982; Qizilbash, 1982). Invasion is described as T1 if the submucosa, but not the muscularis mucosae is invaded; T2 if the muscularis propria, but not the subserosa is invaded; T3 is invasion of the non-peritonealized pericolic or perirectal tissues. The primary tumour is denoted by the prefix "p"). Mucin production was noted as was the presence of a lymphocyte infiltrate and the

presence of signet cells in which intracellular mucin pushes the nucleus to one side. It was not possible to identify the regional lymph nodes in the specimens and no data have been included on lymph node metastases.

Antibody production has been shown to be a very diverse phenomenon. Relatively simple methods have been devised for producing clones of cells which make a single antibody. This can be applied to the production of monoclonal antibodies. The mouse spleen is a rich source of antibody-forming cells, and fused with an appropriate cell makes a hybrid line. Eventually, single clones of hybrid cells can be grown in tissue culture. Relatively large amounts of antibodies can be made. These antibodies are, of course, identical in structure and affinity for antigen. In a research tool, monoclonal antibodies provide a remarkable asset. Assuming that the cells from which they are derived are immortal, then the antibodies derived from this process over a period of time should be identical.

These techniques have been used to produce monoclonal antibodies which react with markers expressed on the surface of lymphocytes. The seminal experimental work involved mice. Cantor and Boyse (1975) described the Ly series of lymphocyte differentiation antigens which were used to demonstrate the existence of T-cell subpopulations or subsets bearing distinct surface phenotypes. Subsequently, spleen cells from mice were immunized with rat thymocyte membranes with the production of several monoclonal

### 5.3. T LYMPHOCYTE SUBSET ANALYSIS

#### 5.3.1. Development of the techniques

The monoclonal antibody revolution has given rise to many diverse applications. Milstein and Kohler (1975) devised methods for producing clones of cells which make a single antibody. This can be achieved by inoculating and immunising mice with cells or cell fragments from another species e.g. rat or human. The mouse spleen cells, normal antibody-forming cells, are fused with an appropriate B-cell myeloma tumour line. Eventually, single clones of hybrid cells can be grown in tissue culture. Relatively large amounts of antibodies can be made. These antibodies are, of course, identical in structure and affinity for antigen. As a research tool, monoclonal antibodies possess a remarkable asset. Assuming that the cell lines from which they are derived are immortal, then the antibodies derived from this process over a period of time should be identical.

These techniques have been used to produce monoclonal antibodies which react with markers expressed on the surface of lymphocytes. The initial experimental work involved mice. Cantor and Boyse (1975) described the Ly series of lymphocyte differentiation antigens which were used to demonstrate the existence of T-cell subpopulations or subsets bearing distinct surface phenotypes. Subsequently, spleen cells from mice were immunised with rat thymocyte membranes with the production of several monoclonal

antibodies (Williams, 1977). Two relevant antibodies were studied, these were called W3/13 and W3/25. Labelled lymphocytes were analysed using a fluorescence activated cell sorter. It was found that monoclonal W3/13 labelled all thoracic duct T-lymphocytes, but not B-lymphocytes. Monoclonal antibody W3/25 labelled a large proportion, but not all, of T-lymphocytes. It was anticipated that using the fluorescence activated cell sorter it would be possible to separate these subpopulations of lymphocytes and study their function. Milstein's group reported work on the W3/25 antibody labelled cells (White, 1978). They found that W3/25 labelled 80% of rat thymocytes, but only 52-58% of thoracic duct lymphocytes. When B-cells were excluded only 71-75% of the remainder were labelled. The fluorescence activated cell sorter allowed separation of these two cell fractions (W3/25 positive and W3/25 negative).

They described two immune functions which were associated with the W3/25 positive subsets. Firstly, helper cell activity for an anti-hapten plaque forming cell response was confined to the labelled population and secondly graft-versus-host activity was likewise confined to the labelled subset. The unlabelled subset mediated an allogeneic suppressive effect. The MRC OX8 monoclonal antibody was found to label a significant subpopulation of rat thymocytes and all W3/25 -ve peripheral T lymphocytes (Brideau, 1980). Barclay (1981) used MRC OX 8 and W3/25 monoclonal antibodies on cryostat sections of rat spleen and lymph nodes. He found that both these populations were present throughout the T-dependent areas. In the B areas there were only scattered T cells which were largely W3/25 positive cells. When rat spleen lymphocytes and

BCG-activated cells were labelled and tested for natural killer (NK) activity it was found that rat natural killer cells were heterogeneous in their surface antigen expression (Cantrell, 1981). The test used was  $^{51}\text{Cr}$  release assay. Subpopulations of rat NK cells express the OX8 and W3/13 defined antigens, but not the W3/25 defined antigen.

It was known that T cell-deprived animals failed to reject allografts, but the mechanism of this effect was unknown. Dallman (1982) reported a series of experiments which examined the roles of lymphocyte subsets in rats bearing skin allografts. Monoclonal antibodies W3/25 and OX 8 were used to deplete thoracic duct lymphocytes of one or other of these subsets. The residual cells were then injected into syngeneic T cell-deprived rats which had been given allogeneic skin grafts. OX 8 labelled cells had no effect on graft rejection, but removal of the W3/25 labelled cells prolonged graft survival. They examined the thoracic duct lymphocytes of adult thymectomised, lethally irradiated, bone marrow restored rats. A small proportion of these cells were labelled with OX8 antibody. These cells had non-specific cytotoxic activity. These cells, although labelled with OX8, were considerably larger than normal lymphocytes. This was determined by their scatter profile on the fluorescence activated cell sorter.

The authors also cited a personal communication from Cantrell that natural killer cells are larger than normal lymphocytes. It was suggested therefore that the nonspecific cytotoxicity obtained from adult thymectomised, lethally irradiated, bone marrow restored rat

thoracic duct lymphocytes was derived from OX 8 positive natural killer cells.

### 5.3.2. Lymphocyte subset functions in rodents

The functions of W3/25 and OX8 T lymphocytes are summarised in the following table:

Function	Monoclonal antibody		Reference
	W3/25	OX8	
Helper activity for B cells	+	-	\$
Helper activity for T cells	+	-	*
Proliferation in MLR	++	+	*
Graft-vs-host reactivity	+	+	\$
Graft-vs-host disease	++	+	&
Suppression of antibody	-	+	\$
Suppression of antibody	-	+	\$
formation in allogeneic interactions			
Cytotoxic T (cell) precursor	-	+	£
Cytotoxic (effector) T (cells)	-	+	£

Key to references: \$ = White (1978), Brideau (1980), Mason (1980);

\* = Mason (1981); & = Mason (1981b); £ = Dallman (1982).

### 5.3.3. T lymphocyte subset enumeration procedures

#### Blood sampling

All rats were anaesthetised using the open ether method using diethyl ether (May & Baker Pharmaceuticals Ltd, UK). Blood samples were obtained from the tail vein or from the jugular vein after surgical exposure of this vessel in the root of the neck. Terminal samples were obtained by direct cardiac puncture. About two millilitres of blood were placed in sample tubes (Becton Dickinson: Oxford, UK) containing sodium heparin. The tubes were then gently agitated.

#### Lymphocyte separation

Using a polypropylene syringe (Becton Dickinson: Oxford, UK) and hypodermic needle (Becton Dickinson: Oxford, UK) 2ml of Ficoll-Hypaque density gradient (Pharmacia: Milton Keynes, UK) was placed into a 10ml polystyrene centrifuge tube (Sterilin Ltd: Teddington, UK). The rat whole blood was layered carefully onto the surface of the Ficoll-Hypaque. The tubes were then centrifuged at 450g for 30 minutes at 20°C. The plasma was removed carefully using a sterile glass Pasteur pipette (Alpha Laboratories Ltd, Gillingham,



UK). The plasma was discarded. The milky interface between the Ficoll Hypaque and plasma contains monocytes and lymphocytes. This interface was removed using a pipette and placed in separate test tubes. To these tubes was added 10ml of fresh RPMI culture medium (Flow Laboratories: Irvine, UK). They were then centrifuged at 250g for 10 minutes at 4°C. The supernatant was removed and a known quantity of fresh RPMI was added to the pellet of cells lying in the bottom of the tube. This volume varied according to the volume of whole blood in the original sample. This facilitated dilution of the lymphocytes. 20 ul was removed from the suspension to count the cells present. The suspension was centrifuged at 250g for 10 minutes at 4°C.

A portion of the isolated cells was used to prepare a microscope slide for differential analysis, using a cytocentrifuge (Shandon Cytospin 2 - Shandon Southern Products Ltd: Runcorn, UK) to assess the purity of the lymphocyte preparation. The proportion of contaminating polymorphonuclear cells was less than 5%. The cells were counted using a Coulter Counter (Coulter Electronics Ltd, Luton, UK). The number of cells in the pellet could then be calculated. The cells were then resuspended at a specific cellularity for the T cell counts. This was  $4 \times 10^6$ /ml. Four samples were then prepared. In one of these polypropylene centrifuge tubes (Sarstedt: Leicester, UK) was placed 2ml of phosphate buffered saline (PBS)/10% fetal calf serum (Flow Laboratories: Irvine, UK) to which 400ul of the cell suspension were added. The tube was centrifuged at 300g for 10 minutes at 4°C. The supernatant was discarded and 400ul of PBS/10% FCS was added to the

cell pellet. The cells were resuspended and 100ul of this suspension were transferred into each of the three remaining test tubes which contained the monoclonal antibodies.

The monoclonal antibodies used were (vide infra):

W3/13 for total T lymphocytes (Sera-Lab Ltd: Crawley Down, UK)

W3/25 for helper T lymphocytes (Sera-Lab Ltd)

OX8 for suppressor/cytotoxic lymphocytes (Sera Lab Ltd).

The three sample tubes aforementioned contained 10ul of monoclonal antibody solution, 90ul of PBS/10%FCS and 100ul of cells suspended at a concentration of  $4 \times 10^6$ /ml. This left 100ul of cell suspension in the control tube. The four tubes were placed in an ice bath for one hour. They were shaken at 15 minute intervals. Then 2ml of PBS/10%FCS were added to each tube. They were centrifuged for 10 minutes at 300g at 4°C.

This was repeated. Two separate tubes had been prepared. The first contained 45ul of anti-mouse IgG labelled with flouroscein (Miles Scientific: Slough, UK) and 405 ul of PBS/10%FCS. This was mixed thoroughly before use using a rotamix. The second tube contained 40ul rat serum and 460ul PBS/10%FCS. After the prepared lymphocytes had been washed twice and the supernatant removed, to each of the four tubes were added 50ul of anti-mouse IgG and 50ul of the rat serum in medium. All four tubes were placed in an ice bath for for 30 minutes and were shaken every 10 minutes.

The tubes were removed from the ice bath and 2ml of PBS/10%FCS were

added and they were centrifuged for 10 minutes at 300g at 4°C. The supernatant was removed and this process was repeated.

#### Storage of labelled lymphocytes

After the previous step, 100uL of 4% paraformaldehyde (pH>8) (Taab Laboratories Ltd: Reading, UK) was added to each sample tube. The samples were stored wrapped in foil in the fridge at 4°C until counting which was done in batches within a maximum time of two weeks. It has been shown fixation in paraformaldehyde solution does not affect flow cytometric analysis of cells previously stained with fluorescein conjugated antibodies (Lanier and Warner, 1981). The maximum tested storage time of fixed cells labelled in this way was 2 months without significant alterations in light scatter or fluorescence properties. Glutaraldehyde fixation was associated with high immunofluorescence backgrounds. This method was used routinely in our laboratory using rat lymphocytes and, therefore, validation was not performed.

## Monoclonal antibodies

### W3/13 - monoclonal antibody against rat T lymphocytes

This is a mouse IgG antibody which is specific for polymorphonuclear cells, all thymocytes and T lymphocytes, haemopoietic stem cells and plasma cells but not B lymphocytes.

### W3/25 - monoclonal antibody against rat T helper cells

This is a mouse IgG antibody which is specific for most thymocytes, helper T lymphocyte subset and macrophages. No other cell are known to be recognised.

### OX 8 - monoclonal antibody against rat T cells (non-helper subset).

This is a mouse IgG antibody which labels most thymocytes and the peripheral T cell sub-set which includes cytotoxic and suppressor T cells. The helper T cell sub-set is not labelled. Some NK (natural killer) cells are also labelled.

### F.I.T.C. conjugated anti mouse IgG.

This conjugate is prepared in the rabbit and is specific for mouse IgG. The product used is derived from highly purified IgG fraction and high purity fluorescein isothiocyanate.

In the preliminary experiments we found that there seemed to be a degree of non-specific binding of the various monoclonal antibodies which had been purchased as ascitic fluid preparations. This was avoided when the more expensive supernatant preparations were used instead.

### Analysis of T lymphocyte subsets

Initially the samples were counted using a light microscope. The number of cells with full immunofluorescence were counted in ultraviolet light in one view of the eyepiece. Then the total number of lymphocytes were counted in one view with normal light. Several views were counted until a total of at least 200 lymphocytes had been counted.

Subsequently for the experiments described in this thesis, flow cytometric analysis was used to analyse the T lymphocyte subsets. The Becton Dickinson FACS 440 was used. This equipment was maintained and operated by staff of the Department of Haematology, University of Wales College of Medicine. For each sample of labelled cells the machine counted approximately 5000 lymphocytes. It counted the number of these cells which fluoresced, therefore the proportion of labelled cells could be calculated. This process was repeated for the four samples (three labelled and one unlabelled control) prepared from each rat.

Comparison of groups of lymphocyte subsets

The results of the experiments involving enumeration of T lymphocyte subsets are expressed as means of the proportions of all T lymphocytes which have been labelled with a particular monoclonal antibody. The correct statistical method for comparing means of proportions involves a process called partitioning of chi square (Dr. T Peters, personal communication). However, because the cell sorter counts a constant number of cells per batch (5,000) it can be shown that the calculation involves a formula which is equivalent to the standard Student's t test. The following worked example demonstrates the method and compares T-helper cell populations of rats with advanced cancer with those of age-matched normal rats.

DATA: % T helper cells per 5000 cells counted.

	normal	cancer	
	28.5	46.0	
	34.3	37.7	
	36.7	36.7	
	21.2	42.6	
	14.0	40.6	
	18.7	35.9	
	23.1	30.0	
	23.1	25.8	
mean	24.95	36.84	unpaired t-test:
SD	7.73	6.49	t = -3.332 for 14 degrees of freedom,
SE	2.73	2.30	p (two-tailed) = 0.005

Chi squared method.

Normal rats.

animal	1	2	3	4	5	6	7	8	total
% Th	28.5	34.3	36.7	21.2	14.0	18.7	23.1	23.1	199.6
% non-Th	71.5	65.8	63.3	78.8	86.0	81.3	76.9	76.9	600.4
total	100	100	100	100	100	100	100	100	800

Cancer rats.

animal	9	10	11	12	13	14	15	16	total
% Th	46.0	37.7	36.7	42.6	40.6	35.9	30.0	25.8	295.3
% non-Th	54.0	62.3	63.3	57.4	59.4	64.1	70.0	74.2	504.7
total	100	100	100	100	100	100	100	100	800

The difference between the two groups (normal and cancer) is represented by a Chi squared, 1 degree of freedom (2 x 2) table:

199.6	295.3
600.4	505.7

However, ALL differences between rats could be represented by a chi squared on 15 degrees of freedom directly from a 2 x 16 table:

Chi squared, 15 degrees of freedom =

$$\frac{28.5^2}{100} + \frac{34.3^2}{100} + \frac{36.7^2}{100} + \dots + \frac{30.0^2}{100} + \frac{25.8^2}{100} - \frac{495^2}{1600} \quad / (0.3093)(0.6907)$$

The differences between rats within the groups are represented by:

Chi squared with 14 degrees of freedom

$$\chi^2_{14df} = \chi^2_{15df} \text{ minus } \chi^2_{1df}$$

$$= 60.196 - 26.429$$

$$= 33.767$$

$$F = (\chi^2_{1/1}) / (\chi^2_{14/14})$$

$$F_{1,14} = 10.95 \quad (0.005 \text{ point} = 11.06)$$

$$p < 0.01$$



#### 5.4. BLOOD TRANSFUSION

##### 5.4.1 Donor rats

Male rats of the highly inbred line Dark Agouti DA (RT-Ia) were used as blood donors throughout the series of experiments. The rats were originally obtained from Harlan Olac (Bicester, UK), and had been specifically bred for this and other work in the Cardiff Royal Infirmary Animal Unit. These rats were maintained under conventional husbandry conditions and fed on a standard pellet diet supplemented with fresh tap water ad libitum. The donor rats were normal and had not been used in previous experiments and had not previously been used as blood donors.

The donor animals were anaesthetised with ether and the thorax swabbed with alcohol. Blood was withdrawn by cardiac puncture using a sterile polypropylene 10mL syringe (Becton Dickinson: Oxford, UK) and a 21 gauge sterile hypodermic needle (Becton Dickinson: Oxford, UK). The donor animals weighed about 350 g and it was usually possible to obtain 8-10ml of blood from each rat. Prior to cardiac puncture a small amount of heparin sodium (Leo Laboratories Ltd: Aylebury, UK) was placed in the syringe to prevent the blood clotting. The concentration of heparin used was about 30 units/ml (the variation was dependent on the amount of blood aspirated). This blood was transfused to the recipients within one hour.

#### 5.4.2. Transfusion procedure

The recipient rats were anaesthetised lightly with ether (May and Baker: UK). They were placed on the operating bench and the tail was swabbed with chlorhexidine solution (ICI Pharmaceuticals: Macclesfield, UK). A sterile 23 guage butterfly needle (Becton Dickinson: Oxford, UK) was inserted into one of the lateral tail vein about two centimetres distal to the base of the tail. Blood or saline (Travenol Laboratories Ltd: Thetford, UK) was then injected slowly, the needle withdrawn and pressure applied to the site of venepuncture. For the tumour model, the volume injected was 1.5 ml in all cases. Three attempts were made to enter the tail vein and if venepuncture was unsuccessful a cut-down was performed on the jugular vein. A small incision was made above the left clavicle initially to expose the jugular vein. After gentle blunt dissection of the subcutaneous fat and incision of the deep fascia this vein is easily identified as it passes proximally beneath the pectoral muscle. Pressure on the pectoral muscle itself dilates the vein and the vessel can easily be entered using a 25 guage hypodermic needle.

Injecting saline is the easier manoeuvre as a "backflash" of blood is seen on entering the vein. When blood is injected this does not happen so the vessel must be well visualised before the first attempt is made. On withdrawing the needle in this position it was important to apply pressure to achieve haemostasis. The wound was closed with a continuous 4/0 chromic catgut suture (Ethicon:

Edinburgh, UK). The rats were inspected later to look for haematoma formation (only two rats developed haematomas which necessitated withdrawal from the experiment).

## 5.5 INDOMETHACIN

Indomethacin was donated by Merck Sharp & Dohme Research Laboratories.

Over one week the amount of drinking water consumed by experimental rats was measured. The indomethacin was dissolved in 2mL of ethanol and subsequently in 10 litres of tap water to give an approximate of indomethacin of 2mg/kg/day. The drinking bottles containing the indomethacin solutions were changed three times a week.

### 5.5.1 Animal anaesthesia procedure

Rats were premedicated using xylazine (Rhone-Mérieux, Bury St. Edmunds) 0.2 mg/kg body weight by intramuscular injection into the right hind leg. The drug for premedication the amount of anaesthesia required to induce anaesthesia is reduced by approximately 50%. Anaesthesia was induced using Hypnorm (Janssen Pharmaceutical Ltd, Oxford) 0.33 mL per kg body weight by intramuscular injection into the other hind leg. One mL of Hypnorm contains 0.335mg fentanyl citrate and 10mg flunitrazepam. Fentanyl is an analgesic of the morphine type and flunitrazepam is a neuroleptic of the benzodiazepine group.

## 5.6. PERITONEAL MACROPHAGE MODEL

### 5.6.1. Animals

Inbred male Lewis (RT11) weighing 200-250g (Bantin and Kingman, UK) were used as transfusion recipients and also syngeneic donors.

Inbred adult male DA (RT1a) rats (Bantin and Kingman, UK) were used as allogeneic blood donors. The animals were housed in standard laboratory cages in groups of four or five with a 12h: 12h light-dark cycle. The rats were fed water and pelleted rat food ad libitum.

### 5.6.2. Blood transfusion procedure

Donor rats were premedicated using acepromazine (C-Vet, Bury St Edmunds) 0.125 mg/kg body weight by intramuscular injection into the hind limb. Using this drug for premedication the amount of barbiturate necessary to induce anaesthesia is reduced by approximately one third. Anaesthesia was induced using Hypnorm (Janssen Pharmaceutical Ltd, Oxford) 0.33 mL per kg body weight by intramuscular injection into the other hind leg. One mL of Hypnorm contains 0.315mg fentanyl citrate and 10mg fluanisone. Fentanyl is an analgesic of the morphine type and fluanisone is a neuroleptic of the butyrophenone group.

The thorax of the animal was swabbed with alcohol and a direct cardiac puncture performed using a sterile polypropylene 10 mL syringe (Becton Dickinson: Wembley, UK) with a sterile disposable 21g needle (Becton Dickinson: Wembley, UK).

Citrate-phosphate-dextrose (CPD) solution was used as anticoagulant and was prepared by dissolving 26.03g trisodium citrate (dihydrate) (Sigma: Poole, UK), and 3.27g citric acid (monohydrate) (Sigma: Poole, UK), and 2.22g sodium dihydrogen phosphate (monohydrate) (Sigma: Poole, UK), and 25.5g dextrose (Sigma: Poole, UK) to one litre of distilled water. The solution was sterilised by filtration through a 0.22µm filter (Millipore: London, UK). The blood was stored in sterile polypropylene tubes (Costar: Cambridge, UK) containing 20% CPD. The whole blood was either transfused within the next 90min or stored for 24hr at 4°C. Serum was prepared by adding 3-5mL blood to sterile polystyrene tubes (Sterilin: Teddington, UK). The blood was allowed to clot at room temperature for 2 hours and then stored at 4°C for a further 20 hours when the serum was removed using a sterile plastic Pasteur pipette (Alpha: UK) and transferred to a 10mL polystyrene centrifuge tube (Sterilin: Teddington, UK) and centrifuged at 1500g for 30 minutes at room temperature. The serum was transferred to sterile polypropylene tubes. Two control solutions were used to transfuse the rats, one consisting of 250µL CDP and 1mL NaCl 0.9% (Antigen Ltd, Eire), and the second being NaCl 0.9% only.

Recipient rats were anaesthetised using the same regime used for the donor rats. Transfusions consisted of 1mL of either fresh blood,

stored blood, serum, CPD/NaCl, or NaCl. After skin disinfection with ethanol, transfusions injected into a peripheral vein using a sterile 2mL polypropylene syringe (Becton Dickinson: Wembley, UK) and a sterile 23 gauge hyodermic needle (Becton Dickinson: Wembley, UK). If a lateral tail vein could not be entered then the dorsal penile vein or the long saphenous vein on a hind limb was used. The animals were allowed to recover and returned to the cages to be reviewed later to check their recovery.

#### 5.6.3 Elicitation of peritoneal macrophages

Three days after transfusion the rats were given an intraperitoneal injection of sterile 4mL bovine brain heart infusion broth (Gibco: Paisley, UK). The broth was prepared by dissolving 38g of broth powder in 1L of distilled water and subsequent sterilisation by autoclave. The broth was stored in sterile polystyrene containers (Sterilin: Teddington, UK).

#### 5.6.4. Macrophage harvesting

Seven days after transfusion the rats were anaesthetised as before and killed by cardiac air embolus. The abdominal wall was swabbed with ethanol and a midline skin incision made with a sterile scalpel (Swann-Morton: Sheffield, UK). A small incision was made in the muscle wall to expose the peritoneum. Without opening the peritoneal cavity, 10mL of sterile, cooled phosphate buffered

saline(PBS): pH 7.3 containing 5mM ethylene-diaminetetracetate (EDTA) (Sigma: Poole, UK) was injected into the peritoneal cavity. After one minutes mixing, the peritoneal cavity was incised and the lavage fluid aspirated using a sterile plastic Pastuer pipette. The fluid was transferred to a sterile polystyrene 10mL centrifuge tube (Sterilin: Teddington, UK) which was placed in an ice bath.

#### 5.6.5. Preparation of the macrophages for culture

The peritoneal fluid was centrifuged at 300g (4°C) for 10 minutes. The following cell preparation was performed in a flow cabinet (Envair: Rossendale, UK). The supernatant was discarded and 10mL PBS pH 7.3 added to the resuspended cell pellet and the suspension centrifuged at 300g (4°C) for 10 minutes. This step was repeated once and the supernatant discarded. Contaminating red blood cells were lysed hypotonically by adding 5 mL 0.2% NaCl to the resuspended cell pellet followed after one minute by 5 mL 1.6% NaCl and the suspension centrifuged at 300g (4°C) for 10 minutes and the supernatant was then discarded. The cells were resuspended in 5mL PBS and 400uL removed for counting and staining.

#### 5.6.6. Counting cells and assessing viability

A counting chamber with improved Neubauer ruling was used (Johnstone and Thorpe, 1987). A 200uL sample of cell suspension was added to a polypropylene test tube (Sarstedt: Leicester, UK) and to this was

added 200uL of 0.4% trypan blue stain (Sigma: Poole, UK) added, vortexed and incubated for 5 minutes in an ice bath. A 20uL sample was added to the counting chamber under the cover slip and the cells allowed to settle for one minute. The cells contained in 25 large squares were counted, representing a volume of 100uL, using a laboratory tally counter. Viable cells exclude trypan blue whereas non-viable cells take up the stain. The total of cells contained in the original 5mL is calculated.

The remaining 4.6mL of cell suspension was centrifuged at 300g (4°C) for 10 minutes and the supernatant discarded. The cells were resuspended in Medium 199 (Flow Laboratories: Irvine, UK) with Earle's balanced salt solution and supplemented with penicillin/streptomycin, L-glutamine and 7.5% fetal calf serum (all Flow Laboratories: Irvine, UK) to achieve a final concentration of  $10^6$  cells per mL.

#### 5.6.7. Non-specific esterase stain

The morphologic identification of mononuclear phagocytes in a mixed cell population is not always reliable. The purity of the monocyte preparation may be gauged by a number of procedures. A simple and reliable technique is the the histochemical stain for non-specific esterase (Hayhoe and Quigley, 1980). The cytoplasm of monocytes and macrophages will stain diffusely with this stain, but neutrophils fail to stain (Figure 5.1). Some lymphocytes do stain, but in a small granular (2-3 granules per cell) distribution rather than



diffusely.

Cells were incubated with alpha-naphthyl butyrate in the presence of the stable diazonium salt, pararosaniline hydrochloride. Enzymatic hydrolysis of ester linkages by the alpha-naphthyl butyrate esterase, liberated free naphthyl compounds (at pH 5.8-6.2), which complex with the diazonium salt to form highly coloured red/brown deposits at the sites of enzyme activity.

## Procedure

### a) Preparation of slides

Initially microscopic slides were prepared by placing a drop of cell suspension on a glass slide and forming a smear by drawing the drop across the slide with a second slide. This method proved to be unsatisfactory for two reasons. Firstly, there was an uneven distribution the various cell types: all cells were concentrated near the edges of the smear and non-specific esterase positive cells were found in greater numbers at the beginning and at the end of the cell smear. This made counting the proportions of cell types inaccurate. Secondly, the smear method resulted in disruption of many cells: the macrophages seemed to be particularly fragile. This method was abandoned in favour of the cytocentrifuge method (Table 5.6.1).

TABLE 5.6.1

Comparison of rat peritoneal macrophage enumeration techniques using the non-specific esterase stain.

Technique	(n)	total cells mean (SD)	macrophages % mean (SD)	CV*
smear + lab prepared reagents	31	13.1 (6.53)	33.8 (15.9)	47%
cytocentrifuge + lab prepared reagents	27	14.9 (6.6)	41.0 (11.3)	28%
cytocentrifuge + stain kit	22	10.5 (2.42)	31.0 (6.85)	22%

\*CV = coefficient of variation for the macrophage counts.

The first set of data was derived from a pilot study which preceded the main experiment.

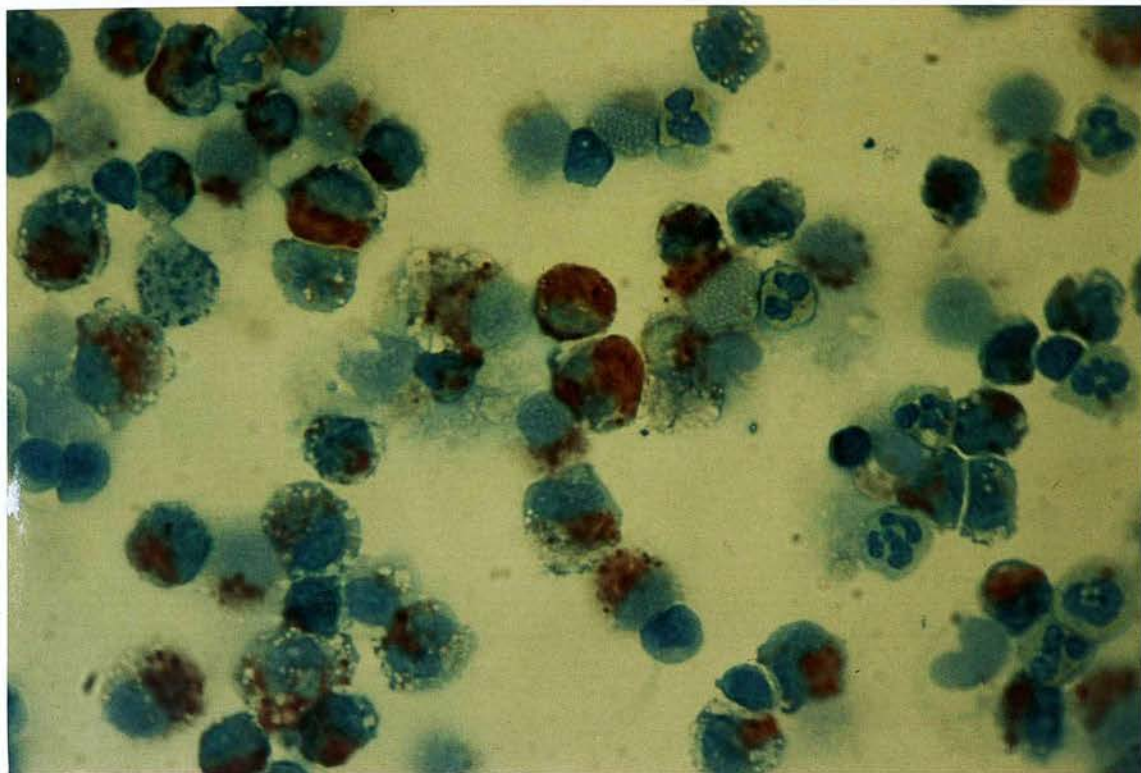
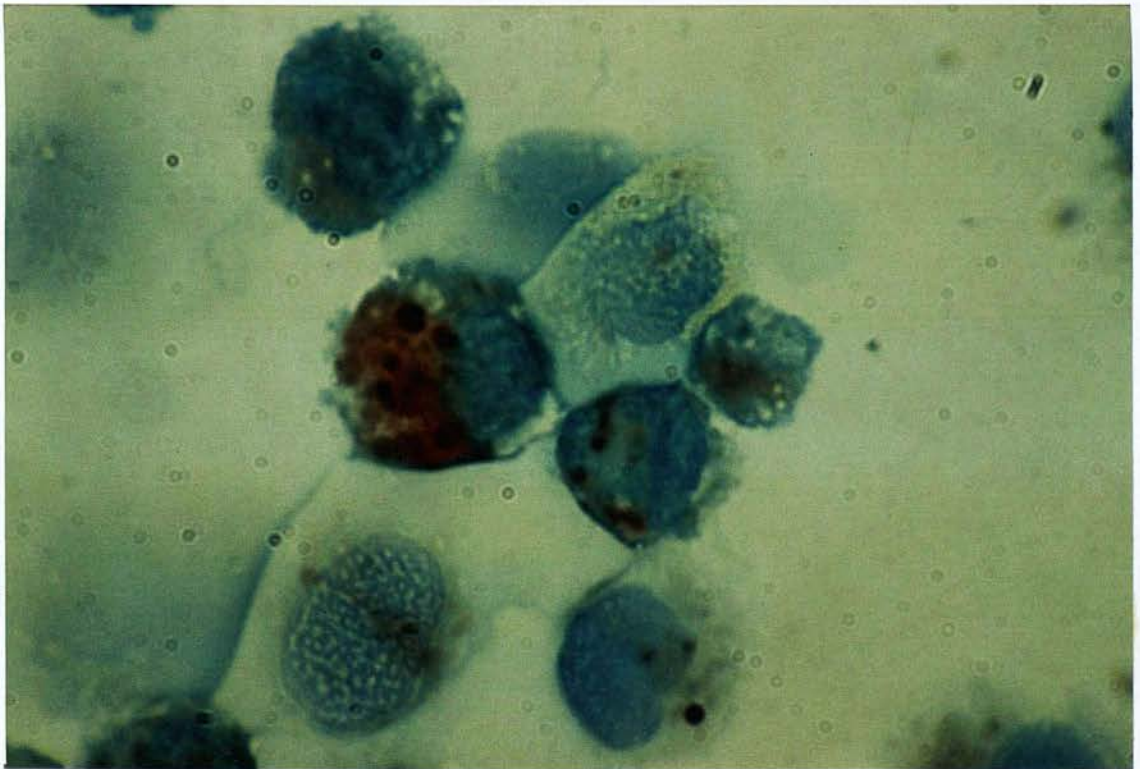


Figure 5.1

Non-specific esterase staining of a peritoneal cellular suspension. Monocytes take on a characteristic red brown stain in the cytoplasm. Lymphocytes may stain in a small 'dot' area in the cytoplasm. Magnification x 400.

Figure 5.2

Non-specific esterase staining of a peritoneal cellular suspension. Monocytes take on a characteristic red brown stain in the cytoplasm. Lymphocytes may stain in a small 'dot' area in the cytoplasm. Magnification x 1000.





Microscope slides were prepared for use using a cytocentrifuge (Shandon Cytospin II: Shandon Southern Products Ltd, Runcorn, UK). The optimal number of cells for the slide preparation was found to be  $5 \times 10^5$  in 100uL PBS. Therefore, 200uL from the 5mL suspensions of the harvested rat peritoneal cells was diluted with with PBS (1:5, v:v) and placed in polypropylene test tubes (Sarstedt: Leicester, UK). The cytocentrifuge was loaded with labelled empty glass microscope slides (Chance Proper Ltd: Worley, UK) which had been washed previously. The cytocentrifuge was set to run for 4 minutes at a speed of 1000rpm with low acceleration. The cell suspension was vortexed to ensure mixing and 100uL added to the cytocentrifuge carriers and centrifuged onto the slides. The slides were removed and air-dried for at least 15 minutes prior to fixation.

b) Fixation

The preparations were fixed using a glutaraldehyde fixative solution. Glutaraldehyde fixative solution was prepared by adding 9mL 25%(w/v) glutaraldehyde solution (Sigma: Poole, UK) to 21mL deionized water and 45mL reagent grade acetone (Rathbone Chemicals: Walkerburn, UK). The solution was mixed well and stored in an airtight amber glass bottle in the freezer, at below  $-10^{\circ}\text{C}$ . The fixative solution was placed in a pre-cooled Coplin jar and kept capped in the freezer at below  $-10^{\circ}\text{C}$ . The fixative solution was mixed vigorously

before use and returned to the storage container immediately after use. The air dried slides were fixed for exactly 5 minutes in the freezer, at below  $-10^{\circ}\text{C}$ . The slides were removed from the fixative and rinsed in gently running deionized water for 30 seconds. The slides were allowed to air dry for at least 15 minutes prior to proceeding with the stain.

c) Staining reaction

Evaluation of staining was carried out using reagents purchased separately (Sigma: Poole, UK) and using an alpha-naphthyl butyrate non-specific esterase stain kit (Sigma: Poole, UK). Using the former method, the staining patterns were unreliable with marked variation in intensity of the staining patterns. Using the kit, greater consistency was achieved, but this still did not reach total consistency and all slides were prepared and stained in duplicate.

The reagents were bought as a kit: alpha naphthyl butyrate esterase kit (Sigma: Poole, UK). The phosphate buffer solution consisted sodium and potassium phosphates (not specified) and had a concentration of  $0.067\text{ mol/L}$ , pH 7.7 at  $25^{\circ}\text{C}$ . Microbial growth was retarded by filtration through 0.22 micron filter unit (Flow Laboratories: Irvine, UK). Before use 40mL buffer was warmed to  $37^{\circ}\text{C}$ . To 1.5mL pararosaniline solution (40g/L in 2mol/L hydrochloric acid) was added 1.5mL 4% sodium nitrite solution. This was vortexed vigorously, and stood for 5 minutes before it was added to the

buffer. Then 5mL of alpha-naphthyl butyrate solution (2.4g/L, solubilizers and methanol warmed to 37°C prior to use) was added. The final solution was mixed well and poured into two Coplin jars. The slides were added and incubated for one hour at 37°C. After one hour the slides were removed and the used solution discarded. The slides were rinsed for 3 minutes in deionized water and allowed to air dry for at least fifteen minutes before counterstaining. The slides were counter-stained for 5 minutes in methylene blue. This solution was prepared immediately before use by adding 5mL methylene blue (1.4%(w/v) in 95% ethanol) to 45mL deionized water. Counter-staining was done using Giesma's stain originally, but the methylene blue supplied with the kit proved to have superior visual qualities.

d) Evaluation of slides

Slides were evaluated using 400x/1000x magnification. Two staining patterns were recognised. One was a focal (dot) type characterized by 1-2 centrally located areas of activity or a diffuse cytoplasmic red-brown reaction. The latter pattern represents monocytes and macrophages. Neutrophils do not stain and lymphocytes may stain with the focal type of pattern. On each slide 200 cells were counted and the proportion of non-specific esterase positive cell calculated.

#### 5.6.8. Macrophage culture

The methodology which had been established previously by the author was extended using microtitre culture techniques (Beavis et al, 1989 Appendix 2). After counting and resuspension of the harvested in culture medium cells to a concentration of  $10^6$  cells/mL, 300uL aliquots were placed in triplicate in 96 well polystyrene tissue culture plates (Flow Laboratories: Irvine, UK). These were incubated at  $37^{\circ}\text{C}$  in 5% carbon dioxide, /95% air for one hour. The culture medium was then removed from each well by aspiration through a sterile 21 guage hypodermic needle. Fresh M-199 culture medium was added to the wells and the plates incubated for a further 20 hours. A 250uL sample of each well supernatant was removed and stored in 96 well polystyrene trays and sealed with a self-adhesive plate sealer (Flow Laboratories: Irvine, UK). The samples were stored overnight at  $-20^{\circ}\text{C}$  to be assayed the next day or, otherwise, stored at  $-70^{\circ}\text{C}$ .



Comparison of adherent and non-adherent cell populations (non-specific esterase stain).

Peritoneal cell suspensions were obtained from six untreated control rats and prepared according to the preceding protocols and resuspended in culture medium at a concentration of  $10^6$  cells/mL. Replicate samples of each were placed in a 96 well polystyrene tissue culture plate. After one hour, the supernatants were removed and placed in polypropylene test-tubes. Adherent cells were removed from the wells by adding PBS containing 5mM EDTA, a polypropylene pipette tip was used to dislodge the cells and the sealed plates were shaken mechanically. Microscopic examination of the tissue culture plate monolayers before and after supernatant removal indicated that about one quarter of the adherent cells had not been removed. Cyto- centrifuge preparations of the thoroughly vortexed samples were made immediately and stained using the non-specific esterase technique (Table 5.6.2). In all samples at least 200 cells were counted.

Table 5.6.2

Comparison of adherent and non-adherent cells

	non-specific esterase stain +ve	
	mean	SD
adherent cells (n=6)	67%	7.8%
non-adherent cells (n=6)	0.6%	0.5%

## 5.7. RADIOIMMUNOASSAY

The levels of prostaglandin E2 of the supernatant samples were determined using a high affinity rabbit polyclonal anti-PGE2 radioimmunoassay and iodinated PGE2 tracer purchased as a kit from Du Pont UK Ltd (Stevenage, UK) using modifications to the suggested protocol.

### 5.7.1. Introduction

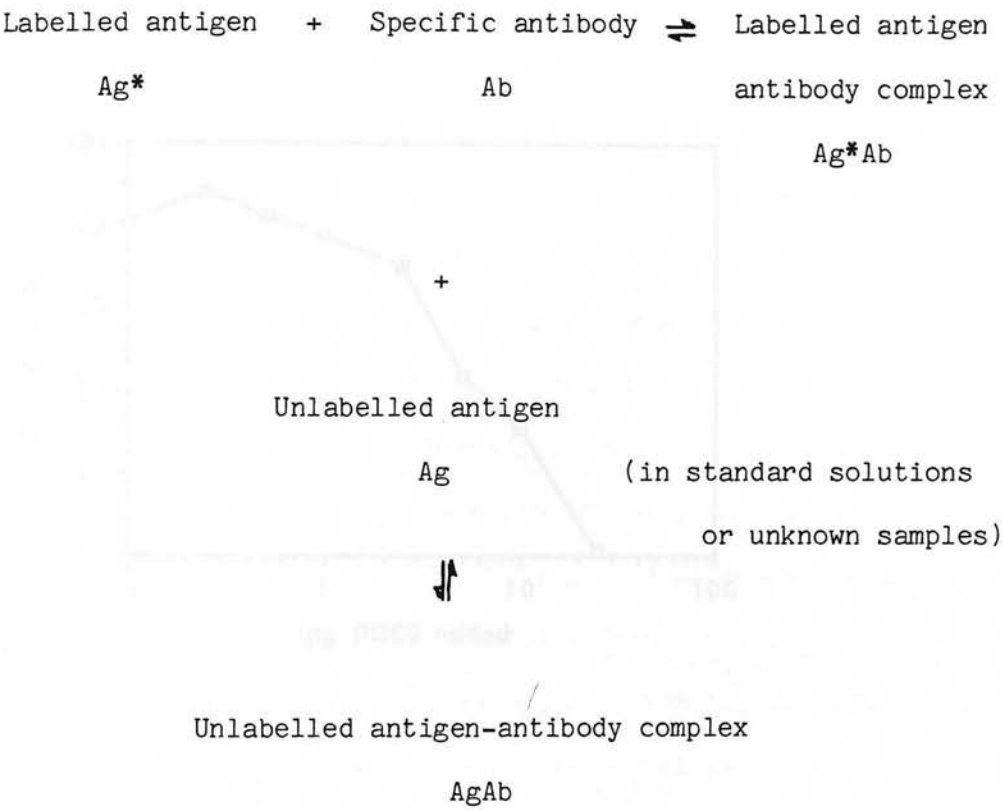
Prostaglandin E2 (PGE2) has been detected in plasma and urine and in a variety of human tissues (Samuelsson et al, 1975). However, the level of PGE2 is often very low ( $<10\text{nM}$ ) and requires sensitive procedures for accurate measurement. The early methods of bioassay and gas chromatography/mass spectrometry were either too insensitive, non-specific, or cumbersome for routine use (Samuelsson et al, 1970; Ferreira et al, 1967; Green et al, 1973). The development of specific antisera resulted in radioimmunoassays which approached the sensitivity required (Levine, 1973; Jaffe et al, 1974). However, the relatively low specific activity of the tritiated PGE2 used in these assays limited the sensitivity that could be obtained. High specific activity, iodinated, prostaglandin tracers were developed and used to increase the sensitivity of these radioimmunoassay systems (Macclouf et al, 1976).

### 5.7.2. Principle of the method

The prostaglandin assay used in the current experiments is based on the use of an iodinated prostaglandin E2 as tracer and rabbit anti-prostaglandin E2 as the antiserum (specific antibody).

The basic principle of the radioimmunoassay involves competitive binding, where a radioactive antigen competes with a non-radioactive antigen for a fixed number of antibody binding sites. When unlabelled antigen from standards or samples and a fixed amount of tracer (labelled antigen) are allowed to react with a constant and limiting amount of antibody as the amount of unlabelled antigen is increased (Figure 5.3). The binding activity of the antibody is quantitated using results obtained for the standards which are used to construct a standard (dose-response) curve from which the concentration of the unknowns are determined by interpolation. A typical binding curve is indicated (Figure 5.4)

FIGURE 5.3 Radioimmunoassay principle



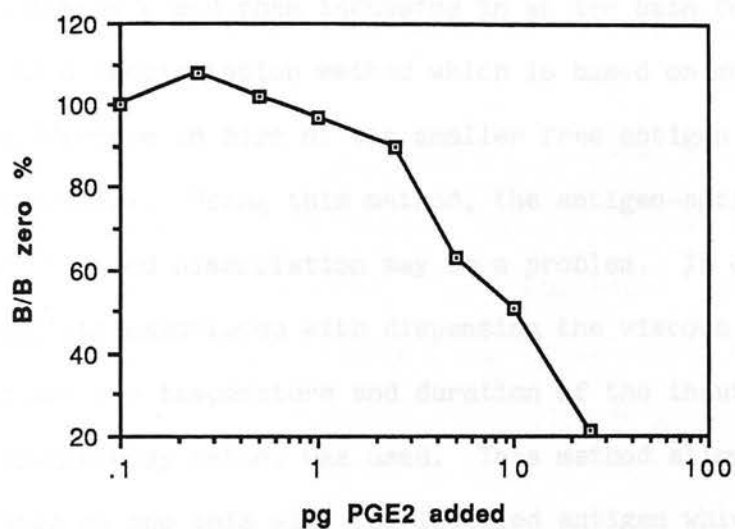


Figure 5.4

Typical standard curve for the prostglandin E2 radioimmunoassay.

The methodology of the assay then deviates from the Du Pont kit method in that separation of the antibody-antigen complexes from free antigen is achieved by an incubation with normal rabbit serum and donkey antirabbit serum. The kit method achieves this separation with polyethylene glycol. One mL of the cold precipitating fluid is added to the tubes containing sample, tracer and antibody - and then incubated in an ice bath for 30 minutes. This is a precipitation method which is based on molecular size i.e. the difference in size of the smaller free antigen and the larger bound complex. Using this method, the antigen-antibody reaction is reversible and dissociation may be a problem. In order to avoid the variability associated with dispensing the viscous precipitating fluid and the temperature and duration of the incubation, the double-antibody method was used. This method allows a large lattice to build up and this with the labelled antigen which is bound to the first antibody can be precipitated (Hunter, 198 ). In general, the disadvantages of the double antibody method relate to the increased cost and to the need to use the second antibody in relatively large concentrations.

After centrifugation, the supernatant containing the unbound antigen is decanted, and the pellet containing the antibody-antigen complex is counted in a gamma counter. Results obtained for the standards are used to construct a standard (dose-response) curve from which the unknowns are read by computerized interpolation.

### 5.7.3. Reagent description and preparation

- a) PGE2 [ $^{125}\text{I}$ ] Tracer (Du Pont UK Ltd; Stevenage, UK)

The tracer concentrate contained  $< 2\mu\text{Ci}$  of PGE2, [ $^{125}\text{I}$ ], in 0.75mL of acetonitrile. Stored at  $-20^{\circ}\text{C}$ , the tracer was stable for at least one month after receipt in the laboratory. The half-life of this isotope is 80 days.

For use in the assay, an appropriate aliquot of tracer concentrate was diluted 1:20 (v:v) in assay buffer in a polypropylene tube. The tracer was transferred using a positive displacement pipettor (Oxford, Eire) and disposable polypropylene pipette tips (Oxford, Eire).

- b) PGE2 Antibody (Du Pont UK Ltd; Stevenage, UK)

The lyophilized rabbit anti-PGE2 was stored at  $2-8^{\circ}\text{C}$  and was stable for two months after receipt. It was reconstituted with 13mL of assay buffer for use. This solution was stored at  $2-8^{\circ}\text{C}$  and was stable for two months.

- c) PGE2 Standard Concentrate (Du Pont UK Ltd; Stevenage, UK)

This solution contained 100 ng/mL of prostaglandin E2 in acetonitrile. Immediately before use in the assay, an aliquot of the stock solution was diluted to prepare standards (vide infra). Stored at  $-20^{\circ}\text{C}$ , the tracer was stable for at least two months after receipt in the laboratory. Care was taken to avoid evaporation of the solvent and resultant concentration of the standard.

Unnecessary exposure to air and ambient temperature was avoided and after use the vials were capped and immediately returned to the freezer.

d) Assay Buffer (Du Pont UK Ltd; Stevenage, UK)

This solution consisted of 0.9% NaCl, 0.01M EDTA, 0.3% bovine gamma globulin, 0.005% Triton-X-100, 0.05% sodium azide, 0.0255M NaH<sub>2</sub>PO<sub>4</sub>.H<sub>2</sub>O, 0.0245M Na<sub>2</sub>HPO<sub>4</sub>.7H<sub>2</sub>O, pH 6.8. Stored at at 2-8°C and was stable for two months.

Additional buffer solution was made as necessary to the same composition (all materials - Sigma: Poole, UK). The assay buffer contained additives (Triton-X-100 and gamma globulin) which minimize the non-specific adsorption of prostaglandin E<sub>2</sub> to the wall of the test tubes used in the assay.

e) Donkey Anti-Rabbit Serum (Scottish Antibodies Production Unit, Carlisle, UK)

This antibody forms complexes with the the anti-PGE<sub>2</sub> antibody in the assay. The donkey anti-rabbit serum (DARS) was stored at -20°C, and the same batch was used throughout the experiments. The DARS was diluted 1:8 (v:v) to obtain a working stock.

Optimization of the DARS concentration in this assay had been performed previously. Different dilutions of DARS were tested to find the concentration that gave approximately 50% binding in the zero standard. For the batch used in this assay a 1:8 (v:v) dilution was used.



- f) Normal rabbit Serum (Scottish Antibodies Production Unit, Carlisle, UK)

This antibody aids formation of large complexes. The normal rabbit serum (NRS) was stored at  $-20^{\circ}\text{C}$ , and the same batch used throughout the experiments. The NRS was diluted 1:140 (v:v) to obtain a working stock.

#### 5.7.4. Sample handling

Sample handling was performed using the Oxford Sampler System range of positive displacement pipettes (Oxford, Eire). The pipette tips were disposable and made of polypropylene (Oxford, Eire). The performance characteristics of these pipettes were assessed by weighing consecutive dispensed aliquots on a microbalance. The 50-200uL pipette was the principal pipette used in this assay and the performance characteristics are tabulated in Table 5.7.1.

TABLE 5.7.1

Sample handling - performance characteristics of 50-200uL pipette

Volume set uL	number of measurements	Mean weight ug	Standard deviation	coefficient of variation
50	20	47.680	0.7010	2.6%
100	20	96.340	1.3830	1.4%
150	20	145.995	1.0267	0.7%

This pipette was also tested by dispensing 100uL aliquots of [14C] phosphatidylcholine into test tubes and counting the scintillation in a beta counter. By this method a coefficient of variation of 3.717% was observed.

		Concentration
a		(22/0.1uL)*
a	0.1uL (100uL) standard + 1.5uL assay buffer	100
b	0.1uL of dilution a + 1.5uL assay buffer	25
c	0.4uL of dilution b + 0.6uL assay buffer	10
d	0.1uL of dilution c + 0.4uL assay buffer	5
e	0.4uL of dilution d + 0.4uL assay buffer	2.5
f	0.4uL of dilution e + 0.4uL assay buffer	1.25
g	0.4uL of dilution f + 0.4uL assay buffer	0.5
h	0.4uL of dilution g + 0.4uL assay buffer	0.25
*this concentration represents actual mass added to the assay tube.		
Dilutions b to h were used for the standard curve.		

5.7.5.      Procedure

a)    Preparation of Prostaglandin E2 Working Standards

An aliquot of the PGE2 standard concentrate (tube "a" in the dilution scheme - Table 5.7.3) was diluted with assay buffer in order to prepare a series of working prstaglandin E2 standards (tubes b-h in the dilution scheme). The dilution to cover a standard curve range of 0.25pg to 25pg added (per 0.1mL) is shown in Table 5.3. Working standards were freshly prepared immediately before use and were placedin 3.5mL polypropylene test tubes (Sarstedt: Leicester, UK).

TABLE 5.7.3  
Dilution Scheme for Prostaglandin E2 Standards

Tube		Concentration (pg/0.1mL)*
a	0.1mL (100uL) standard + 1.9mL assay buffer	500
b	0.1mL of dilution a + 1.9mL assay buffer	25
c	0.4mL of dilution b + 0.6mL assay buffer	10
d	0.4mL of dilution c + 0.4mL assay buffer	5
e	0.4mL of dilution d + 0.4mL assay buffer	2.5
f	0.4mL of dilution e + 0.6mL assay buffer	1.0
g	0.4mL of dilution f + 0.4mL assay buffer	0.5
h	0.4mL of dilution g + 0.4mL assay buffer	0.25

\*This concentration represents actual mass added to the assay tube.  
Dilutions b to h were used for the standard curve.

b) Radioimmunoassay procedure

All reagents were prepared as previously described, mixed and allowed to equilibrate to room temperature.

Polypropylene 3.5mL centrifuge tubes (Sarstedt, Leicester, UK) were labelled in duplicate for total counts, blank each standard and each sample. 100uL of each diluted standard (b-h) were put into tubes in duplicate and 100uL of each diluted sample were pipetted in duplicate into the appropriate tubes. 100ul of tracer solution was added to each tube and the solutions vortexed at 2200 cycles per minute for 5 seconds (Sarstedt CM-9; Leicester, UK). 100uL of antiserum were pipetted into all tubes containing standards and samples and vortexed for 5 seconds.

The samples were incubated for two hours at room temperature. Then, 100uL of normal rabbit serum were added to the the blank, standard and sample tubes and mixed. Next, 100uL of donkey anti-rabbit serum were added and vortexed. The tubes were capped and placed in the fridge to incubate at 4°C for 18 hours. The tubes were then centrifuged in a refrigerated centrifuge at 1,550 x g for 30 minutes at 4°C. With the exception of the total counts tubes, the supernatants were decanted and blotted on absorbant paper. All tubes were then counted in a gamma counter.

c) Radioimmunoassay controls

The assay system included the following controls:

TOTAL COUNTS - containing 100uL tracer only

BLANK- containing 200uL buffer, 100uL tracer

0 STANDARD - containing 100uL buffer, 100uL tracer, 100uL  
anti-PGE antibody

STANDARD SAMPLES - seven concentrations in duplicate  
(Table 5.7.3)

INTRA-ASSAY CONTROLS - containing standard samples  
appropriate to the mid-point of the linear portion of the  
radio-ligand binding curve (5pg PGE<sub>2</sub>). At least six  
control tubes were included distributed throughout the  
assay, but upto twelve tubes were included in the larger  
assays (>50 samples).

#### 5.7.6. Gamma counter

Bound radiolabelled PGE<sub>2</sub> was analysed using the LKB Wallac Multigamma 1260 gamma counter was used (LKB: Stockholm, Sweden). This was a microcomputer controlled gamma counter which simultaneously counts 12 samples of a gamma emitting isotope.

#### 5.7.7. Calculation of unknowns

The microcomputer used a spline function program to calculate the concentration of the unknowns. The spline function method provides a means of describing the standard curve in radioimmunoassay which allows calculation of the concentration of unknown samples with a high degree of precision. The mathematical procedures relating to spline function have been described in detail in the literature (Grenville, 1967; Reinsch, 1967; Reinsch, 1971; Hayes, 1974).

All counts were corrected to counts per minute (CPM). The counts were averaged for each set of duplicates. The net counts for all standards and samples were calculated by subtracting from each the average blank counts.

The normalized percent bound (%B/B0) for each standard and sample was calculated according to the formula:

$$\%B/B0 = \frac{\text{net CPM of standard or sample}}{\text{net CPM of "0" standard}} \times 100$$

The computer plotted %B/B0 for each standard using a semilogarithmic graph versus the corresponding amounts of of PGE2 added in picograms. The PGE2 in each sample was determined (by the computer) by interpolation from the standard curve. The sample values were then corrected for dilution to determine the original concentration in the supernatant sample.

0.01	0.01	Arachidonic acid	<0.01
0.02	0.02	GMPPGalpha	<0.01
0.04	0.04	PGE1	<0.01
0.08	0.08	PGE2	<0.01
0.16	0.16	GMPPGalpha	<0.01
0.32	0.32	Linoleic acid	<0.01
0.64	0.64	PGE2	<0.01

5.7.8 Cross-reactivity of the assay

The manufacturer's manual gives data concerning cross-reactivity (Table 5.7.4).

TABLE 5.7.4

Cross-reactivity of PGE2 antibody used in assay.

Compound	% cross-reactivity	Compound	% cross-reactivity
PGE2	100	Arachidonic acid	<<0.01
PGE1	3.7	DHKF2alpha	<<0.01
DHKPGE2	0.4	PGA1	<<0.01
PGA2	0.4	PGB2	<<0.01
PGF1	0.03	6KPGFalpha	<<0.01
Thromboxane B2	0.02	Linoleic acid	<0.001
PGF2	<0.01	PGD2	<0.001



5.7.9.      Reproducibility of the assay

Intra-assay reproducibility was evaluated by running multiple replicates of samples with of known concentration of PGE2 standard concentrate dilutions (TABLE 5.7.5).

TABLE 5.7.5

Intra-assay variation (5pg PGE2 added to tube)

Assay No.	Replicates (n)	Corrected counts/min	SD	Coefficient of variation
1	8	2793	214	13%
2	8	3463	306	9%
3	6	1860	106	6%
4	6	2766	186	7%
5	8	2767	98	3.5%
6	6	2764	187	7%
7	12	1976	137	7%

5.7.10. Inter-assay variation

Inter-assay reproducibility was evaluated on the one occasion when two separate batches of tracer were available, but using the same batches of antibody and standard PGE2 concentrate (Table 5.7.6).

TABLE 5.7.6

Inter-assay reproducibility

Tracer	Replicates	PGE2 added	RIA concentration, mean	SD
A	6	5pg	4.22pg	1.08
B	7	5pg	4.80pg	0.84

5.7.11      Effects of storage conditions on sample PGE2  
concentration

PGE2 standard concentrate was diluted in assay buffer. Aliquots of PGE2 solution containing 5pg/100uL were added to polystyrene 96 well culture plates and also to sterile polypropylene 2mL storage vials. The samples were stored at -20°C for 24 hours. They were then thawed and assayed for PGE2 (Table 5.7.7). There was no significant difference between the two methods of storage.

TABLE 5.7.7

Effects of storage conditions on sample PGE2 assay

Container	Replicates (n)	PGE2 added	Corrected counts/min	SD
Polystyrene	6	5pg	2767	104
Polypropylene	8	5pg	2763	205

#### 5.7.12. Matrix correction

The samples from the cell culture supernatants include diluted fetal calf serum. To investigate the possibility of fetal calf serum affecting the binding curve, standard curves were performed with two concentrations of fetal calf serum and compared with a standard curve derived from samples in normal buffer.

Fetal calf serum was added to buffer to obtain concentrations equivalent to a 1:50 (v:v) and 1:100 (v:v) dilutions of supernatant samples. These dilutions were required in concurrent experiments not described here. The final concentration of fetal calf serum in the assay buffer were therefore 0.15% and 0.075% respectively. The resultant binding curves are shown in Figure 5.5. It will be seen that there is a clear shift to the left with a fetal calf serum concentration of 0.15%. The effect of 0.075% FCS is less clear, but a similar shift was found when the experiment was repeated (data not shown). In all subsequent assays, the standards were diluted with buffer containing a constant concentration of FCS (0.75%). Supernatant samples were diluted 1:9 (v:v) with normal buffer and further dilutions made with buffer containing 0.75% FCS.

# Standard curves - effect of fetal calf serum

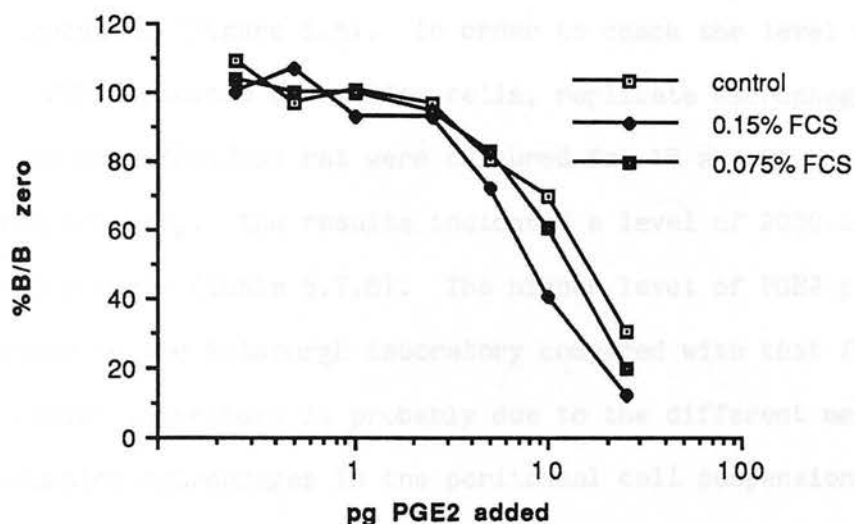


FIGURE 5.5

Standard curves for the PGE2 radioimmunoassay using two different concentrations of fetal calf serum in the standard samples. The third curve is for standard samples diluted in buffer without fetal calf serum.

5.7.13. Macrophage PGE2 production - time course

The time course of this rat peritoneal macrophage PGE2 production had been established previously (Beavis et al, 1989: Appendix 2). PGE2 was generated by resting cultured peritoneal macrophages in a time dependent manner reaching a maximum after 19 hours of incubation (Figure 5.6). In order to check the level of the plateau of PGE2 produced by resting cells, replicate macrophage preparations from an individual rat were cultured for 18 and 24 hours respectively. The results indicated a level of 2000-2500pg per  $10^6$  macrophages (Table 5.7.8). The higher level of PGE2 production found in the Edinburgh laboratory compared with that found in the Cardiff laboratory is probably due to the different method of counting macrophages in the peritoneal cell suspension: the previous method depended on morphological features to identify macrophages and this probably over-estimated the number present.

TABLE 5.7.8

Macrophage PGE2 production after 18 and 24 hours culture

	n	PGE2 production/ $10^6$ macrophages	
18 hour culture	9	2272pg	(SD=1637)
24 hour culture	9	2536pg	(SD=2184)

# **Macrophage PGE2 production time course**

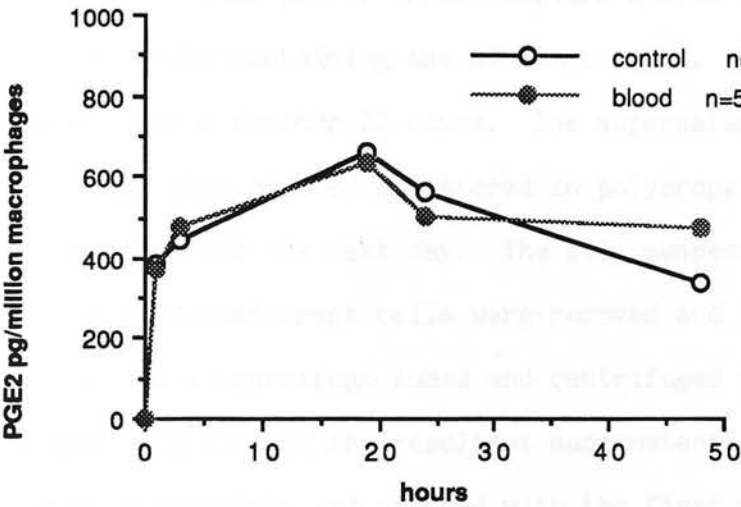


Figure 5.6

Pilot study: macrophage prostaglandin E2 production by normal control rats and by rats transfused with allogeneic blood - time course studies (see Appendix 2).

5.7.14. PGE2 production by adherent and non-adherent cells

Replicate samples of five control rat peritoneal cell suspensions in culture medium were plated out and cultured for one hour.

Supernatants were removed from each well and put in separate empty wells on the same plate. Fresh culture medium was added to the original wells containing the adherent cells. The cells were cultured for a further 20 hours. The supernatants from the adherent cell wells were removed and stored in polypropylene vials and assayed for PGE2 the next day. The cell suspensions from the wells containing non-adherent cells were removed and placed in polypropylene centrifuge tubes and centrifuged at 300g for 10 minutes. Samples of the resultant supernatants were stored in polypropylene vials and assayed with the first set of samples (Table 5.7.9). It should be noted that the non-adherent cell sample includes PGE2 produced by the total cells during the first culture period of one hour (to allow macrophage adherence to take place).

TABLE 5.7.9

PGE2 production by adherent and non-adherent cells

	(n)	mean	SD
adherent cells, PGE2 pg/10 <sup>6</sup> macrophages	5	1213	45
non-adherent cells, PGE2 pg/mL	5	160	35



#### 5.7.15. Statistical analysis

Statistical analysis has been performed using a non-parametric method for paired data (Wilcoxon rank sum test). The reasons for using a paired analysis are twofold, the first was planned and the second became obvious as the experiment progressed. The use of paired analysis was endorsed by the statistical adviser to this part of the project (Dr G Raab). Firstly, within each cycle, each of the two or three subgroups was transfused with fresh blood, stored blood or serum derived from one syngeneic or allogeneic rat. A paired analysis was performed between fresh and stored blood transfusions because both blood units were obtained from the same rat. Secondly, there was an obvious variation in results with the separate experimental groups through the course of the experiment (Table 5.7.10). The experiment was carried out in five cycles: the first two cycles each had two groups of eight rats, while the last three cycles had three groups of eight rats. The PGE<sub>2</sub>/10<sup>6</sup> macrophages of the first two groups were much lower on average than those levels found in the last three groups and this was independent of the macrophage counts. A paired analysis could also be made between the transfused and control rats within subgroups because the cells retrieved were subjected to the same experimental conditions. A sizable inter-assay variation relating to the radio-immunoassay was demonstrated and this may have contributed. There may have been unknown differences in the animals, such as infection causing pre-stimulation of the monocytes.

TABLE 5.7.10

Inter cycle variation in PGE2 production.

Cycle - (date started)	(n)	Total cells mean (SD)	Macrophages % mean (SD)	PGE2, pg mean (SD)
1 (4.12.89)	12	15.0 (7.2)	40.6 (15.1)	2358 (1890)
2 (8.1.90)	15	14.8 (6.2)	42.1 (7.1)	2501 (2089)
3 (22.1.90)	22	10.5 (2.4)	31.0 (6.9)	7410 (4478)
4 (12.2.90)	22	11.6 (2.7)	41.7 (7.1)	4580 (1893)
5 (27.2.90)	22	11.2 (2.9)	39.2 (6.2)	7134 (3917)

Cycle: this consisted of 2 or 3 groups which each contained 8 individual rats each of which seven received a different transfusion and one was untreated. The dates of RIA were - 1: 14.12.89; 2: 17.1.90; 3: 21.2.90; 4: 21.2.90; 5: 7.3.90.

## 6. RESULTS

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6.1. CLINICAL RETROSPECTIVE STUDY

6.1.1. Patients and exclusions

During the period 1977-1980, 314 patients underwent bowel resection for colorectal cancer. After exclusion of patients who had palliative surgery, patients who died during the perioperative period, and patients whose records were incomplete, there were 236 patients left to study (Table 6.1.1).

TABLE 6.1.1

Exclusions

incomplete records	12
perioperative deaths	25
palliative surgery only	41
curative resection	236
total	<u>314</u>

The mean length of follow-up for the whole group (n = 236) was 34.1 months. The length of follow-up in months: mean (SD), was similar in transfused and non-transfused patients: 32.6 (22.7) months versus 37.5 (23.6) months respectively.

6.1.2. Clinical features

The distribution of tumour site in the 256 patients who had curative resections, including abdomino-perineal resections, is shown in Table 6.1.2.

TABLE 6.1.2

Distribution of tumour site in patients having curative resection

tumour site	n (% total)		% transfused
caecum	22	9%	59%
ascending colon	25	11%	68%
transverse colon	13	5%	46%
descending colon	16	7%	56%
sigmoid colon	45	19%	51%
high/mid rectum	38	15%	71%
low rectum	77	30%	91%
total	<u>256</u>	<u>100%</u>	<u>70%</u>

Blood transfusions were given to 165 patients (70%). This group was compared with the group of 71 patients who did not receive blood transfusions (Table 6.1.3).

TABLE 6.1.3

Comparability of patients who received blood and those who did not  
- clinical features.

Variable	Blood transfusions		P <sup>*</sup>
	Yes (n=165)	No (n=71)	
sex: n, %			
male	88, 53%	37, 53%	
female	77, 47%	34, 47%	N.S.
age: years - mean (SD)	68.4 (11.2)	66.7 (11.2)	N.S.
presentation for surgery: n, %			
emergency	11, 7%	12, 17%	
elective	154, 93%	59, 83%	0.028

\* Student's t test or Chi square test

TABLE 6.1.4

Comparability of patients who received blood and those who did not -  
pathological features.

Variable	Blood transfusions		p*
	Yes (n=165)	No (n=71)	
site of tumour: n, %			
right colon	36, 22%	24, 34%	
left colon and rectum	129, 78%	47, 66%	
adherent tumour: n, %	45, 27%	13, 18%	N.S.
annular tumour: n, %	44, 26%	14, 20%	N.S.
tumour diameter, cms	4.61 (1.59)	4.29 (1.67)	N.S.
- mean (SD)			
clinical stage: n, %			
Dukes B	111, 67%	49, 69%	N.S.
Dukes C	54, 33%	22, 31%	N.S.
histological grade: n, %			
well differentiated	51, 31%	24, 34%	
moderately differentiated	104, 63%	44, 62%	
poorly differentiated	10, 6%	3, 4%	N.S.

\* Student's t test or Chi square test



TABLE 6.1.5

Distribution of surgeons performing the curative resection.

Variable: n, %	Blood transfusions	
	Yes (n=165)	No (n=71)
consultant 1	44, 27%	17, 24%
consultant 2	9, 5%	4, 6%
consultant 3	15, 9%	4, 6%
consultant 4	9, 5%	4, 6%
consultant 5	22, 13%	10, 14%
senior registrars	23, 14%	10, 14%
registrars and SHOs	43, 26%	22, 31%
all consultants	99, 60%	39, 55%
trainees	66, 40%	32, 45%

TABLE 6.1.6

Patients undergoing staged procedures

Variable: n, %	Blood transfusions		p*
	Yes (n=165)	No (n=71)	
one operation	126, 76%	63, 89%	N.S.
two operations	29, 18%	7, 10%	
three operations	10, 6%	1, 1%	

However, significantly more of the transfused patients had a staged resection rather than a single operation compared with non-transfused patients (39/165 versus 8/71;  $p = 0.045$ ). The pathologists reported the number of lymph nodes seen in the resected specimen and counted the number of lymph nodes in which tumour was seen (Table 6.1.7). Lymph node involvement was seen in 54/165 in the transfused group and in 22/71 in the non-transfused group.

TABLE 6.1.7

Lymph node involvement in patients with Dukes C lesions

Variable	Blood transfusions			p*
	Yes (n=54)	No (n=22)		
lymph nodes seen: mean (SD)	7.13 (6.15)	5.59 (3.28)	N.S.	
lymph nodes involved: mean (SD)	2.72 (2.07)	2.18 (1.82)	N.S.	

\* Student's t test.

6.1.3.      Survivorship data

TABLE 6.1.8a

Life table calculations for transfused patients: all deaths during 60 month period following curative resection of colorectal cancer.

time (months)	died	lost	alive	at risk	est. probability death	est. probability survival	percentage survivors
0-6	15	1	165	164.5	0.09	0.91	100 %
7-12	13	4	149	147	0.09	0.91	90.88
13-18	12	7	132	128.5	0.09	0.91	82.84
19-24	16	2	113	112	0.14	0.86	75.11
25-30	14	3	95	93.5	0.15	0.85	64.38
31-36	10	1	78	77.5	0.13	0.87	54.74
37-42	7	4	67	65	0.11	0.89	47.68
43-48	8	3	56	54.5	0.15	0.85	42.54
49-54	5	4	45	43	0.12	0.88	36.30
55-60	1	9	36	31.5	0.03	0.97	32.08
>60			26				31.06

See Figure 6.1

TABLE 6.1.8b

Life table calculations for non-transfused patients: all deaths during 60 month period following curative resection of colorectal cancer.

time (months)	died	lost	alive	at risk	est. probability death	probability survival	percentage survivors
0-6	5	1	71	70.5	0.07	0.93	100%
7-12	7	1	65	64.5	0.11	0.89	92.91
13-18	4	1	57	56.5	0.07	0.93	82.82
19-24	3	1	52	51.5	0.06	0.94	76.96
25-30	6	2	48	47	0.13	0.87	72.48
31-36	3	1	40	39.5	0.08	0.92	63.23
37-42	0	4	36	34	0.00	1.00	58.42
43-48	2	3	32	30.5	0.07	0.93	58.42
49-54	3	3	27	25.5	0.12	0.88	54.59
55-60	1	4	21	19	0.05	0.95	48.17
>60			16				45.63

See Figure 6.1

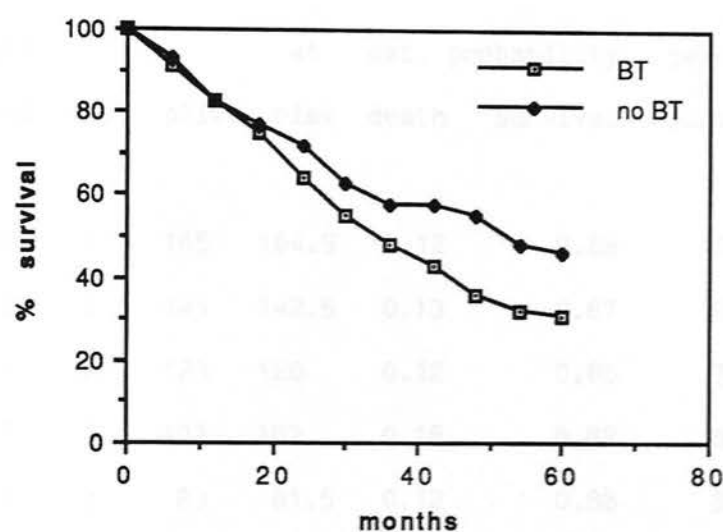


Figure 6.1

Life table analysis 5 year survival curves for patients undergoing curative resection for colorectal cancer.

TABLE 6.1.8c

Life table calculations for transfused patients: recurrence-free survival during 60 month period following curative resection of colorectal cancer.

time (months)	rec/ died	lost	alive	at risk	est. probability death	probability survival	percentage survivors
0-6	20	1	165	164.5	0.12	0.88	100%
7-12	18	3	144	142.5	0.13	0.87	87.84
13-18	14	6	123	120	0.12	0.88	76.75
19-24	18	2	103	102	0.18	0.82	67.79
25-30	10	3	83	81.5	0.12	0.88	55.83
31-36	7	1	70	69.5	0.10	0.90	48.98
37-42	8	4	62	60	0.13	0.87	44.05
43-48	9	3	50	48.5	0.19	0.81	38.17
49-54	3	4	38	36	0.08	0.92	31.09
55-60	1	9	31	26.5	0.04	0.96	28.50
>60			21				25.36

See Figure 6.2

TABLE 6.1.8d

Life table calculations for non-transfused patients: recurrence-free survival during 60 month period following curative resection of colorectal cancer.

time (months)	rec/ died lost		at alive	at risk	est. probability death	est. probability survival	percentage survivors
0-6	7	1	71	70.5	0.10	0.90	100%
7-12	12	1	63	62.5	0.19	0.81	90.07
13-18	3	1	50	49.5	0.06	0.94	72.78
19-24	3	1	46	45.5	0.07	0.93	68.37
25-30	4	1	42	41.5	0.10	0.90	63.86
31-36	3	1	37	36.5	0.08	0.92	57.70
37-42	2	3	33	31.5	0.06	0.94	52.96
43-48	2	2	28	27	0.07	0.93	49.6
49-54	1	3	24	22.5	0.04	0.96	45.92
55-60	2	4	20	18	0.11	0.81	43.88
>60			14				39.01

See Figure 6.2

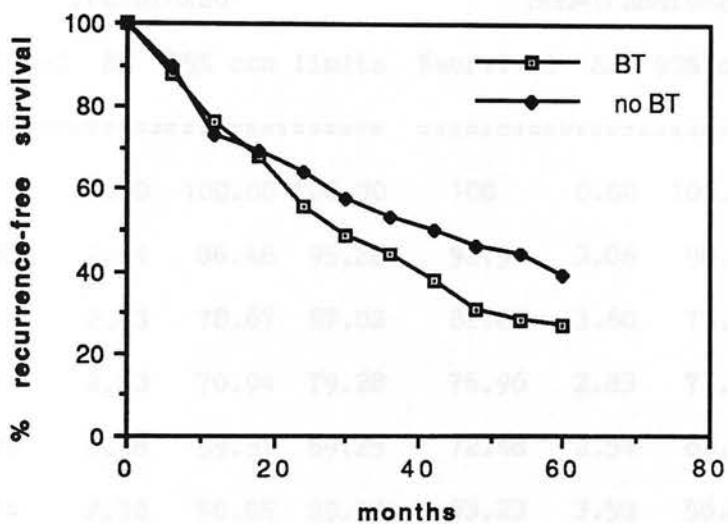


Figure 6.2

Life table analysis 5 year recurrence-free survival curves for patients undergoing curative resection for colorectal cancer.



TABLE 6.1.8e

Comparison between survival rates of transfused and non-transfused patients following curative resection of colorectal cancer with standard errors (SE) and 95% confidence limits

time	transfused				non-transfused			
months	%survival	SE	95% con	limits	%survival	SE	95% con	limits
	=====				=====			
0-6	100	0.00	100.00	100.00	100	0.00	100.00	100.00
6-12	90.88	2.24	86.48	95.28	92.91	3.06	86.92	98.90
12-18	82.84	2.13	78.67	87.02	82.82	3.60	75.77	89.88
18-24	75.11	2.13	70.94	79.28	76.96	2.83	71.42	82.50
24-30	64.38	2.48	59.51	69.25	72.48	2.51	67.55	77.40
30-36	54.74	2.38	50.08	59.39	63.23	3.53	56.31	70.14
36-42	47.68	2.08	43.59	51.76	58.42	2.67	53.20	63.65
42-48	42.54	1.83	38.95	46.13	58.42	0.00	58.42	58.42
48-54	36.30	2.04	32.30	40.29	54.59	2.62	49.46	59.72
54-60	32.08	1.77	28.60	35.55	48.17	3.48	41.34	55.00
>60	31.06	1.00	29.09	33.02	45.63	2.47	40.80	50.47

The logrank test gives a chi-squared value of 3.25;  $p > 0.05$ .

The Mantel-Haenszel method gives a standardised normal deviate of 1.902;  $p < 0.03$ .

TABLE 6.1.8f

Comparison between recurrence-free survival rates of transfused and non-transfused patients after curative resection of colorectal cancer with standard errors (SE) and 95% confidence limits

time months	transfused				non-transfused			
	%survival	SE	95% con	limits	%survival	SE	95% con	limits
	=====				=====			
0-6	100	0.00	100.00	100.00	100	0.00	100.00	100.00
6-12	87.84	2.55	82.85	92.84	90.07	3.56	83.09	97.05
12-18	76.75	2.44	71.95	81.54	72.78	4.49	63.98	81.57
18-24	67.79	2.25	63.38	72.20	68.73	2.47	63.53	73.20
24-30	55.83	2.56	50.81	60.84	63.86	2.52	58.93	68.79
30-36	48.98	2.03	45.00	52.96	57.70	2.93	51.97	63.44
36-42	44.05	1.77	40.58	47.51	52.96	2.62	47.82	58.10
42-48	38.17	1.93	34.38	41.96	49.60	2.30	45.09	54.11
48-54	31.09	2.13	26.91	35.27	45.92	2.50	41.02	50.82
54-60	28.50	1.43	25.69	31.31	43.88	2.00	39.97	47.79
>60	27.42	1.05	25.36	29.49	39.01	3.25	32.64	45.38

The logrank test gives a chi-squared value of 2.016;  $p > 0.05$ .

The Mantel-Haenszel method gives a standardised normal deviate of 1.514;  $p > 0.06$ .

In order to restrict analysis to patients undergoing an abdominal resection only, the patients who had low rectal lesions treated by abdomino-perineal resection were excluded.

Of the 159 patients in the study group, excluding abdomino-perineal resections, 95 (60%) received one or more transfusions of whole blood or packed red cells. The number of units of blood transfused varied between 1 and 13 with a mean of 3.1 units. The patients who received blood were similar to those who did not when the clinical factors were compared, with the exception of preoperative haemoglobin concentration (Table 6.1.9). The patients who were transfused had a lower preoperative haemoglobin than those who were not. When the pathological features of the two groups were compared, some significant differences were found (Table 6.1.10). The seniority of the surgeon did not influence the transfusion rate. When a consultant performed the resection 58% of patients (n=80) were transfused. When the resection was performed by a junior surgeon 61% of patients (n=79) were transfused. A higher proportion of patients who had staged procedures (n=40) were transfused compared with those who had a primary resection (n=119); (83% versus 52%,  $p<0.002$ ).

TABLE 6.1.9

Comparability of patients who received blood and those who did not - clinical features.

Variable	Blood transfusions		p*
	Yes (n=95)	No (n=64)	
Sex, %			
male	46	48	
female	54	52	N.S.
Age, years - mean (SD)	68.9 (10.1)	66.3 (11.4)	N.S.
Preoperative haemoglobin			
males, g/dl - mean (SD)	12.5 (2.37)	14.1 (2.25)	<0.01
females, g/dl - mean (SD)	12.0 (1.94)	13.6 (1.84)	<0.01
Presentation for surgery, %			
emergency	12	19	
elective	88	81	N.S.

\* Student's t test or Chi square test

TABLE 6.1.10

Comparability of patients who received blood and those who did not - pathological features.

Variable	Blood transfusions		p*
	Yes (n=95)	No (n=64)	
Site of tumour, %			
right colon	38	38	
left colon and rectum	62	62	N.S.
Tumour adherent to	31	14	<0.05
other structures, %			
Annular tumour, %	34	17	<0.05
Tumour diameter, cms	4.0 (1.52)	4.2 (1.62)	N.S.
- mean (SD)			
Clinical stage, %			
Dukes B	72	70	
Dukes C	28	30	N.S.
Histological grade			
well differentiated	36	34	
moderately differentiated	58	61	
poorly differentiated	6	5	N.S.

\* Student's t test or Chi square test

TABLE 6.1.11a

Life table calculations for transfused patients: all deaths during 60 month period following curative resection of colorectal cancer (excluding AP resections).

time (months)	died	lost	alive	at risk	est. probability death	probability survival	percentage survivors
0-6	8	0	95	95.0	0.08	0.92	100%
7-12	7	3	87	85.5	0.08	0.92	91.58
13-18	4	4	77	75.0	0.05	0.95	84.08
19-24	10	2	69	68	0.15	0.85	79.60
25-30	8	3	57	55.5	0.14	0.86	67.89
31-36	7	0	46	46	0.15	0.85	58.11
37-42	5	3	39	37.5	0.13	0.87	49.26
43-48	5	1	31	30.5	0.16	0.84	42.69
49-54	1	6	25	22	0.05	0.95	35.70
55-60	0	3	18	16.5	0.00	1.00	34.07
>60			15				34.07

See Figure 6.3

TABLE 6.1.11b

Life table calculations for non-transfused patients: all deaths during 60 month period following curative resection of colorectal cancer (excluding AP resections)

time (months)	died	lost	alive	at risk	est. probability death	probability survival	percentage survivors
0-6	5	1	64	63.5	0.08	0.92	100%
7-12	6	0	58	58	0.10	0.90	92.13
13-18	3	1	52	51.5	0.06	0.94	82.60
19-24	3	1	48	47.5	0.06	0.94	77.78
25-30	4	2	44	43	0.09	0.91	72.87
31-36	2	1	38	37.5	0.05	0.95	66.09
37-42	2	3	35	33.5	0.06	0.94	62.57
43-48	1	3	30	28.5	0.04	0.96	58.83
49-54	3	3	26	24.5	0.12	0.88	56.77
55-60	1	4	20	18	0.06	0.94	49.82
>60			15				47.05

See Figure 6.3

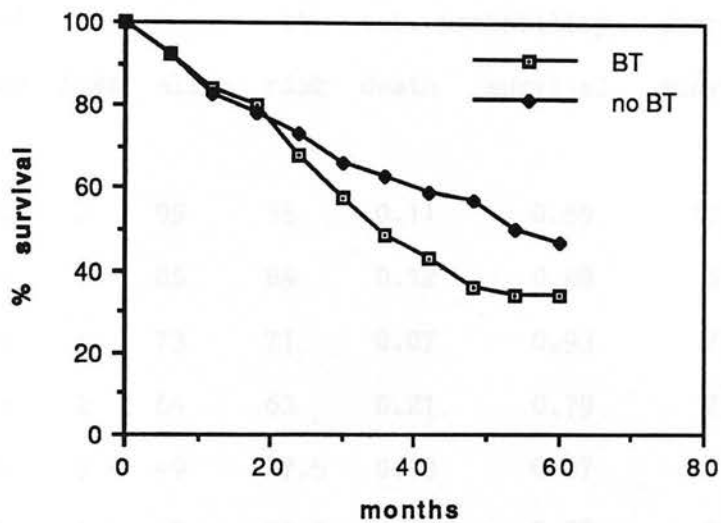


Figure 6.3

Life table analysis 5 year survival curves for patients undergoing curative resection for colorectal cancer (excluding patients who had abdomino-perineal resections).



TABLE 6.1.11c

Life table calculations for transfused patients: recurrence-free survival during 60 month period following curative resection of colorectal cancer (excluding AP resections)

time (months)	rec/ died	lost	alive	at risk	est. probability death	est. probability survival	percentage survivors
0-6	10	0	95	95	0.11	0.89	100%
7-12	10	2	85	84	0.12	0.88	89.47
13-18	5	4	73	71	0.07	0.93	78.82
19-24	13	2	64	63	0.21	0.79	73.27
25-30	6	3	49	47.5	0.13	0.87	58.15
31-36	3	1	40	39.5	0.08	0.92	50.81
37-42	6	3	36	34.5	0.17	0.83	46.95
43-48	4	1	27	26.5	0.15	0.85	38.78
49-54	2	4	22	20	0.10	0.90	32.93
55-60	0	4	16	14	0.00	1.00	29.64
>60			12				29.64

See Figure 6.4

TABLE 6.1.11d

Life table calculations for non-transfused patients: recurrence-free survival during 60 month period following curative resection of colorectal cancer (excluding AP resections)

time (months)	rec/ died	lost	alive	at risk	est. probability death	probability survival	percentage survivors
0-6	7	1	64	63.5	0.11	0.89	100%
7-12	9	0	56	56	0.16	0.84	88.98
13-18	2	1	47	46.5	0.04	0.96	74.68
19-24	3	1	44	43.5	0.07	0.93	71.46
25-30	3	2	40	39	0.08	0.92	66.54
31-36	2	1	35	34.5	0.06	0.94	61.42
37-42	2	3	32	30.5	0.07	0.93	57.86
43-48	2	2	27	26	0.08	0.92	54.06
49-54	1	3	23	21.5	0.05	0.95	49.9
55-60	2	4	19	17	0.12	0.88	47.58
>60			13				41.99

See Figure 6.4

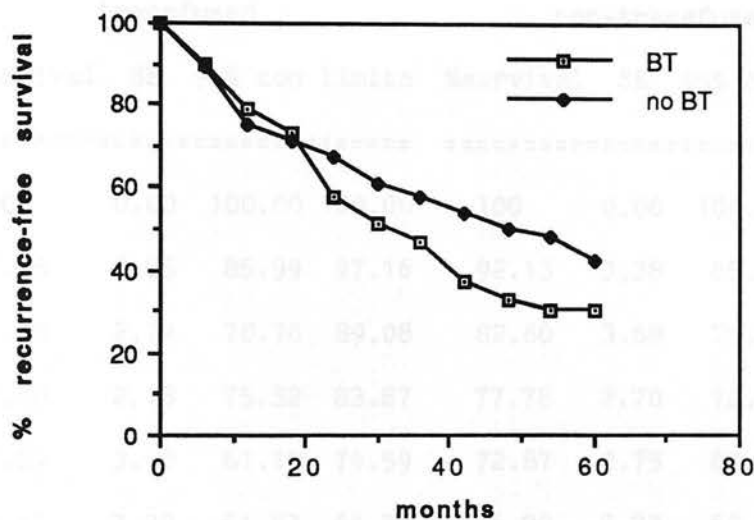


Figure 6.4

Life table analysis 5 year recurrence-free survival curves for patients undergoing curative resection for colorectal cancer (excluding patients who had abdomino-perineal resections).

TABLE 6.1.11e

Comparison between survival rates of transfused and non-transfused patients following curative resection (excluding AP resections) with standard errors (SE) and 95% confidence limits

time months	transfused				non-transfused			
	%survival	SE	95% con limits		%survival	SE	95% con limits	
	=====				=====			
0-6	100	0.00	100.00	100.00	100	0.00	100.00	100.00
6-12	91.58	2.85	85.99	97.16	92.13	3.38	85.50	98.75
12-18	84.08	2.72	78.76	89.08	82.60	3.68	75.38	89.82
18-24	79.60	2.18	75.32	83.87	77.78	2.70	72.50	83.07
24-30	67.89	3.42	61.19	74.59	72.87	2.75	67.49	78.25
30-36	58.11	3.20	51.83	64.38	66.09	3.23	59.77	72.42
36-42	49.26	3.08	43.23	55.29	62.57	2.43	57.81	67.32
42-48	42.69	2.73	37.33	48.05	58.83	2.56	53.81	63.85
48-54	35.70	2.86	30.09	41.31	56.77	2.03	52.79	60.74
54-60	34.07	1.59	30.97	37.18	49.82	3.76	42.45	57.19
>60	34.07	0.00	34.07	34.07	47.05	2.69	41.78	52.32

The logrank test gives a chi-squared value of 2.196;  $p > 0.05$ .

The Mantel-Haenszel method gives a standardised normal deviate of 1.5547;  $p = 0.06$ .

TABLE 6.1.11f

Comparison between recurrence-free survival rates of transfused and non-transfused patients after curative resection (excluding AP resections) with standard errors (SE) and 95% confidence limits

time	transfused				non-transfused			
months	%survival	SE	95% con limits		%survival	SE	95% con limits	
	=====				=====			
0-6	100	0.00	100.00	100.00	100	0.00	100.00	100.00
6-12	89.47	3.15	83.30	95.65	88.98	3.93	81.27	96.68
12-18	78.82	3.16	72.63	85.02	74.68	4.37	66.12	83.24
18-24	73.27	2.39	68.58	77.96	71.46	2.22	67.11	75.82
24-30	58.15	3.74	50.83	65.47	66.54	2.75	61.15	71.92
30-36	50.81	2.80	45.31	56.30	61.42	2.84	55.85	66.98
36-42	46.95	2.14	42.75	51.15	57.86	2.44	53.07	62.65
42-48	38.78	3.03	32.84	44.72	54.06	2.59	48.98	59.15
48-54	32.93	2.70	27.64	38.22	49.90	2.83	44.37	55.44
54-60	29.64	2.21	25.31	33.97	47.58	2.27	43.14	52.03
>60	29.64	0.00	29.64	29.64	41.99	3.72	34.70	49.27

The logrank test gives a chi-squared value of 0.84;  $p > 0.25$ .

The Mantel-Haenszel method gives a standardised normal deviate of 1.271;  $p > 0.11$ .

6.2.1. The carcinogenic effects of dimethylhydrazine in the Sprague Dawley rat (Experiment A)

**Experimental plan**

The aim of this experiment was to evaluate the dimethylhydrazine colonic cancer model in male Sprague Dawley rats. In particular, the time course of the carcinogenesis was analysed to enable future experiments to be planned.

Eighty-nine 8-week old male Sprague Dawley rats were divided into three groups. Group 1 consisted of 36 rats which received 16 weekly injections of dimethylhydrazine at a dose of 16 mg/kg body weight. The solution used included 1.5% EDTA as described in the Methods Section. Group 2 consisted of 36 rats which received 16 weekly injections containing only 1.5% EDTA. The volume of the dose was equal to the volume of dimethylhydrazine injections given to rats in Group 1. Group 3 consisted of normal rats. All rats were kept in plastic cages and fed with the normal diet and given water ad libitum. Each rat had been numbered and was weighed at weekly intervals prior to each injection and at intervals thereafter. Surviving rats were killed at 36 weeks after the first carcinogenic injection. A postmortem examination was performed on these rats and also on rats which had died during the course of the experiments. Rats which were obviously in poor condition were killed. Some representative pathological specimens were examined histologically.

The growth of rats treated with dimethylhydrazine is compared with those in the control group (Table 6.2.1 and Figure 6.5).

TABLE 6.2.1

Growth rate - mean body weight (n; standard deviation) gm.

Group 1 = dimethylhydrazine and EDTA. Group 2 = EDTA alone.

week number	group 1	group 2	p value*
0	201 (36; 47.9)	209 (35; 25.8)	0.37
2	329 (36; 54.2)	317 (35; 31.5)	0.30
4	417 (36; 51.0)	446 (34; 39.4)	0.01
6	483 (36; 52.7)	493 (34; 41.8)	0.39
8	534 (36; 52.2)	565 (34; 47.1)	0.01
10	572 (36; 57.4)	607 (34; 50.3)	0.01
12	598 (36; 60.2)	641 (34; 58.5)	0.0035
14	630 (36; 60.1)	682 (34; 65.8)	0.001
16	648 (36; 61.9)	698 (34; 62.9)	0.0013
24	709 (36; 58.6)	760 (32; 91.2)	0.007
30	690 (23; 74.3)	815 (24; 93.9)	0.0001
36	705 (10; 89.4)	799 ( 7; 97.9)	0.06

\* t test, comparing Group 1 with Group 2.

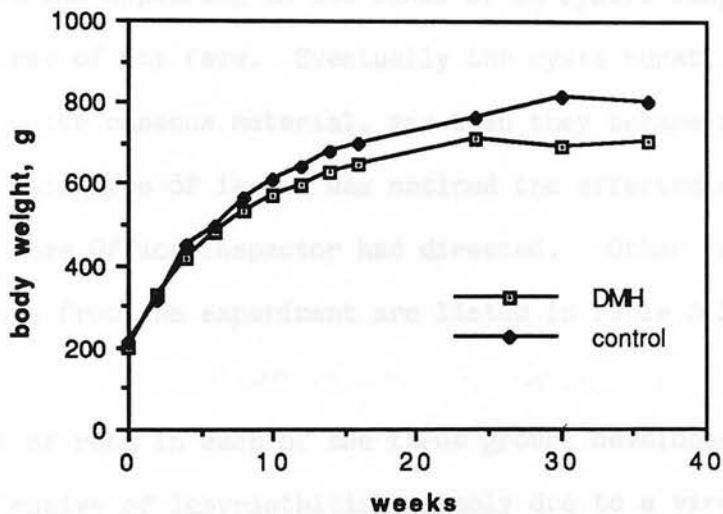


Figure 6.5

Growth curves for male Sprague Dawley rats treated with dimethylhydrazine (DMH) or EDTA (control).



Carcinogenesis.

There were no spontaneous tumours to be found in the rats injected with EDTA or in the group of normal rats. All the rats developed colonic tumours, some with metastatic disease. A few rats developed tumours of the small bowel. Some developed macroscopic tumours involving the external auditory canal. These tumours appeared as papilliform lesions appearing in the canal or as cystic lumps in the preauricular area of the face. Eventually the cysts burst, discharging a white caseous material, and then they became infected ulcers. When this type of lesion was noticed the affected rat was killed as the Home Office Inspector had directed. Other causes for withdrawing rats from the experiment are listed in Table 6.2.2.

A small number of rats in each of the three groups developed signs of ataxia indicative of labyrinthitis probably due to a virus. This presented with the presence of "snuffles" on the nose and an abnormal posture. Affected rats could not maintain balance in what appeared to be a similar manner to humans suffering vertigo. These rats were also killed if these signs persisted.

Spontaneous deaths	1
Deaths due to tumours	4
Deaths due to infection	1
Deaths due to other causes	3
Total spontaneous deaths	14

TABLE 6.2.2

Indications for killing rats during the experiment.

	group 1	group 2	group 3
ataxia	3/13	1/1	1/1
ear tumour	8/13	0/1	0/1
poor condition (cancer)	2/13	0/1	0/1
total number killed	13	1	1

In group 1, fourteen rats died spontaneously during the course of the experiment and the probable causes of death found at post mortem examination are listed in Table 6.2.3. Two rats in group 2 died, one had signs of ataxia and had consolidated lungs at postmortem and the other rats had no obvious cause of death. One rat in group 3 died and this was probably due to pneumonia.

TABLE 6.2.3

Cause of death in rats which died spontaneously (group 1 only).

respiratory infection	1
metastatic disease	4
? colonic infarction	1
unknown, but with bowel tumours	8
total spontaneous deaths	14

The number of rats which were alive 36 weeks after starting dimethylhydrazine injections (group 1) was nine out of the original thirty-six (Table 6.2.4)

TABLE 6.2.4

Survival rates for rats treated with dimethylhydrazine (group 1).

week	number alive	% survival
0	36	100
12	36	100
24	32	89
30	18	50
36	9	25

The distribution of primary tumours found in all rats in group 1 is described in Table 6.2.5. Ten of these rats had metastatic deposits. Six rats had hepatic metastases and three of these rats also had omental or peritoneal deposits of tumour. Four rats had omental secondaries alone and one rat had lung metastases in addition to liver metastases.

TABLE 6.2.5

Primary tumours in rats treated with dimethylhydrazine (n=36)

	rats affected	mean (SD, range) per rat
large bowel	35	4.78 (3.11, 0-12)
right colon	28	1.89 (1.76, 0-7)
left colon	35	2.89 (1.63, 0-7)
small bowel	4	
ear	20	
bilateral ear	5	

6.3. T CELL SUBSETS IN NORMAL AND AGED SPRAGUE DAWLEY RATS  
(Experiment B)

Experimental plan.

The 16-week-old normal male Sprague Dawley rats used in this experiment were also used in Experiment D. Rats aged about 40 weeks were used and these had been some of the normal control animals in Experiment A. The aim of this experiment was to determine normal values for T lymphocyte subpopulations and to examine changes in the various subsets which might occur with age. Both groups of animals had been fed on a normal diet and had not been subject to previous investigation. In the course of this experiment some blood samples were taken which could not be analysed because the blood had clotted during venepuncture. Three samples were from the group of 16 week old rats and one from the older group. These rats were excluded from the study. The two groups are compared in Table 6.3.1.

TABLE 6.3.1

monoclonal antibody	mean % cells labelled (SD)		
	W3/13	W3/25	OX8
16 wk old rats, n=16	65.0 (8.9)	27.2 (8.0)	36.6 (11.5)
40 wk old rats, n=16	65.5 (7.1)	25.5 (6.0)	40.6 (8.1)
p value (t test)	0.86	0.48	0.26

6.4 T LYMPHOCYTE SUBSETS AND THE DIMETHYLHYDRAZINE COLONIC CANCER MODEL.

6.4.1. Do rats with advanced cancer have altered T cell subsets? (Experiment C).

Experimental plan

Peripheral blood lymphocyte subpopulations were enumerated in rats with advanced carcinogenesis. These rats were described in Experiment A and only nine of these were available for testing. One of these samples clotted. Sixteen rats were tested in group treated with EDTA injections (three rats excluded due to samples clotting). Sixteen of the age-matched controls were tested (one sample excluded) as part of Experiment B. These three groups are compared. The eight rats with cancer were compared with the first eight in the consecutive series of EDTA and normal age-matched rats respectively.

TABLE 6.4.1 Rats treated with EDTA with age-matched controls

Comparison of rats with advanced dimethylhydrazine-induced cancer with age-matched controls

	mean % cells labelled (SD)		
monoclonal antibody	W3/13	W3/25	OX8
rats with cancer, n=8	70.1 (7.7)	36.8 (6.5)	45.4 (9.6)
controls, n=8	68.1 (7.5)	25.0 (7.7)	43.7 (7.4)
p value (t test)	0.60	0.005	0.70

TABLE 6.4.2

Comparison of rats with advanced dimethylhydrazine-induced cancer  
with age-matched rats treated with EDTA

monoclonal antibody	mean % cells labelled (SD)		
	W3/13	W3/25	OX8
rats with cancer, n=8	70.1 (7.7)	36.8 (6.5)	45.4 (9.6)
EDTA-treated rats, n=8	74.4 (7.1)	30.1 (6.5)	58.4 (8.8)
p value (t test)	0.26	0.057	0.014

TABLE 6.4.3

Comparison of rats treated with EDTA with age-matched controls

monoclonal antibody	mean % cells labelled (SD)		
	W3/13	W3/25	OX8
EDTA-treated rats, n=15	68.2 (13.5)	27.0 (8.6)	48.7 (13.5)
controls, n=16	65.5 (7.7)	25.5 (6.0)	40.6 (8.1)
p value (t test)	0.50	0.56	0.05

Using the pre-transfusion samples from the rats in Experiments D1, D2, and E, the influence of eight previous weekly injections of dimethylhydrazine on lymphocyte subsets was examined (Table 6.4.4).

TABLE 6.4.4

Comparison of rats after 8 weekly injections of dimethylhydrazine (DMH) with normal age-matched controls

monoclonal antibody	mean % cells labelled (SD)		
	W3/13	W3/25	OX8
DMH rats, n=46	65.3 (11.1)	28.0 (10.0)	34.0 (8.4)
normal rats, n=39	72.5 (10.0)	32.8 (9.3)	37.1 (9.07)
p value (t test)	0.0026	0.022	0.103



6.5. ALLOGENEIC BLOOD TRANSFUSION AND T CELL SUBSETS

6.5.1. T lymphocyte subsets in normal rats treated with blood transfusion (Experiment D1)

Experimental plan.

Male Sprague Dawley rats, aged sixteen weeks received three intravenous transfusions of allogeneic blood from donor DA rats. The volume of each infusion was 1.5 ml and the interval between each was seven days. The blood was transfused within an hour of removal from the donor. Heparin was used for anticoagulation. T lymphocyte subsets in the peripheral blood were assessed before and two weeks after transfusion (Table 6.5.1)

TABLE 6.5.1

Effect of allogeneic blood transfusion on T lymphocyte subsets in normal 16 week old rats - paired data

monoclonal antibody	mean % cells labelled (SD)		
	W3/13	W3/25	OX8
pre-transfusion, n=14	76.7 (8.9)	37.6 (8.4)	36.3 (7.6)
post-transfusion	67.7 (7.1)	32.9 (7.1)	36.5 (11.5)
p value: t test	0.006	0.13	0.97

6.5.2. Effect of normal saline transfusion on T lymphocyte subsets in normal rats (Experiment D2)

Experimental plan.

This experiment was run concurrently with Experiment D1. Male Sprague Dawley rats, aged sixteen weeks received three intravenous transfusions of heparinised 0.9% saline. The volume of each infusion was 1.5 ml and the interval between each was seven days. T lymphocyte subsets in the peripheral blood were assessed before and two weeks after transfusion (Table 6.5.2).

TABLE 6.5.2

Effect of 0.9% saline transfusion on T lymphocyte subsets in normal 16 week old rats - paired data

monoclonal antibody	mean % cells labelled (SD)		
	W3/13	W3/25	OX8
pre-transfusion, n=16	71.6 (8.4)	30.4 (9.8)	40.2 (9.2)
post-transfusion	65.9 (7.2)	28.7 (10.7)	42.9 (13.2)
p value: t test	0.045	0.64	0.55

6.5.3. T lymphocyte subsets in aged rats treated with blood transfusion (Experiment D3)

Experimental plan.

Male Sprague Dawley rats, aged 36 weeks received three intravenous transfusions of allogeneic blood from donor DA rats. The volume of each infusion was 1.5 ml and the interval between each was seven days. The blood was transfused within an hour of removal from the donor. Heparin was used for anticoagulation. T lymphocyte subsets in the peripheral blood were assessed before and two weeks after transfusion (Table 6.5.3).

TABLE 6.5.3

Effect of allogeneic blood transfusion on T lymphocyte subsets in normal 36 week old rats - paired data

monoclonal antibody	mean % cells labelled (SD)		
	W3/13	W3/25	OX8
pre-transfusion, n=6	63.3 (7.8)	23.1 (7.1)	37.5 (7.7)
post-transfusion	69.3 (7.2)	23.8 (5.8)	47.2 (6.4)
p value: t test	0.26	0.84	0.039

6.5.4.      Effect of 0.9% saline transfusions on T lymphocyte  
subsets in aged rats    (Experiment D4)

Experimental plan.

This experiment was run concurrently with Experiment D3. Male Sprague Dawley rats, aged 36 weeks received three intravenous transfusions of heparinised 0.9% saline. The volume of each infusion was 1.5 ml and the interval between each was seven days. T lymphocyte subsets in the peripheral blood were assessed before and two weeks after transfusion (Table 6.5.4).

TABLE 6.5.4

Effect of 0.9% saline transfusion on T lymphocyte subsets in normal 36 week old rats - paired data

monoclonal antibody	mean % cells labelled (SD)		
	W3/13	W3/25	OX8
pre-transfusion, n=6	65.2 (9.2)	24.0 (2.4)	39.5 (8.4)
post-transfusion	69.1 (12.7)	29.2 (4.4)	43.3 (6.9)
p value: t test	0.56	0.029	0.42

6.6. TRANSFUSION AND T CELL SUBSETS IN RATS TREATED WITH DMH

6.6.1. T lymphocyte subsets in rats treated with  
dimethylhydrazine effects of transfusion with blood  
(Experiment E1)

Experimental plan.

Male Sprague Dawley rats, aged sixteen weeks which had already received eight weekly subcutaneous injections of dimethylhydrazine, received three intravenous transfusions of allogeneic blood from donor DA rats. The volume of each infusion was 1.5 ml and the interval between each was seven days. Dimethylhydrazine injections continued during the course of transfusions. T lymphocyte subsets in the peripheral blood were assessed before and two weeks after transfusion (Table 6.5.1)

TABLE 6.6.1

Effect of allogeneic blood transfusion on T lymphocyte subsets in 16-week-old rats which have been treated with dimethylhydrazine - paired data

monoclonal antibody	mean % cells labelled (SD)		
	W3/13	W3/25	OX8
pre-transfusion, n=17	65.8 (10.6)	27.2 (8.0)	35.2 (7.61)
post-transfusion	68.0 (9.1)	24.2 (8.7)	33.9 (9.7)
p value: t test	0.51	0.30	0.67

6.6.2.     T lymphocyte subsets in rats treated with  
dimethylhydrazine effects of transfusion with 0.9% saline  
 (Experiment E2)

Experimental plan.

Male Sprague Dawley rats, aged sixteen weeks which had already received eight weekly subcutaneous injections of dimethylhydrazine, received three intravenous transfusions of heparinised 0.9% saline. The volume of each infusion was 1.5 ml and the interval between each was seven days. Dimethylhydrazine injections continued during the course of transfusions. T lymphocyte subsets in the peripheral blood were assessed before and two weeks after transfusion (Table 6.6.2).

TABLE 6.6.2

Effect of 0.9% saline transfusion on T lymphocyte subsets in 16-week-old rats which have been treated with dimethylhydrazine - paired data

monoclonal antibody	mean % cells labelled (SD)		
	W3/13	W3/25	OX8
pre-transfusion, n=14	69.0 (10.6)	29.9 (12.4)	34.4 (9.3)
post-transfusion	63.3 (10.6)	21.5 (8.7)	35.9 (12.9)
p value: t test	0.12	0.024	0.69

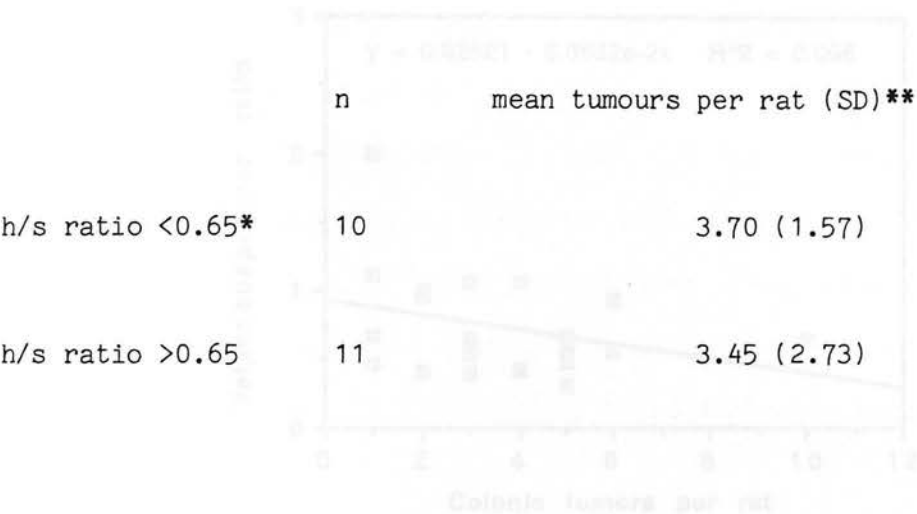
6.7.       RELATIONSHIP OF T CELL SUBSETS TO CARCINOGENESIS  
            (Experiment F)

An experiment had been performed (published elsewhere) to assess the effect of allogeneic blood transfusion on colonic carcinogenesis (Ross et al, 1989 Appendix 1). In this experiment there was no significant difference in the number of histologically proven colonic tumours found in rats transfused with allogeneic blood compared with those transfused with saline. During the course of carcinogenesis venous blood had been sampled to enumerate the T lymphocyte populations. The helper/suppressor ratios for rats in both transfusion groups were calculated and pooled and the median helper/suppressor ratio determined. The new group of rats was divided into two groups about this median. The T cell ratio data were then tabulated with the histopathological data (Table 6.7.1).

The aim was to determine whether rats with low helper/suppressor ratios developed more tumours. Low helper/suppressor ratios may indicate immunosuppression and this state may enhance the process of carcinogenesis. The helper/suppressor ratios were also plotted against the tumour yield of each rat (Figure 6.6).

TABLE 6.7.1.

T lymphocyte (W3/35)/(OX8) helper/suppressor ratio (h/s ratio) and DMH colonic carcinogenesis in Sprague Dawley rats.



\* For the whole group of 21 rats the median h/s ratio was 0.65

\*\* The tumours described are histologically proven adenomas and carcinomas.



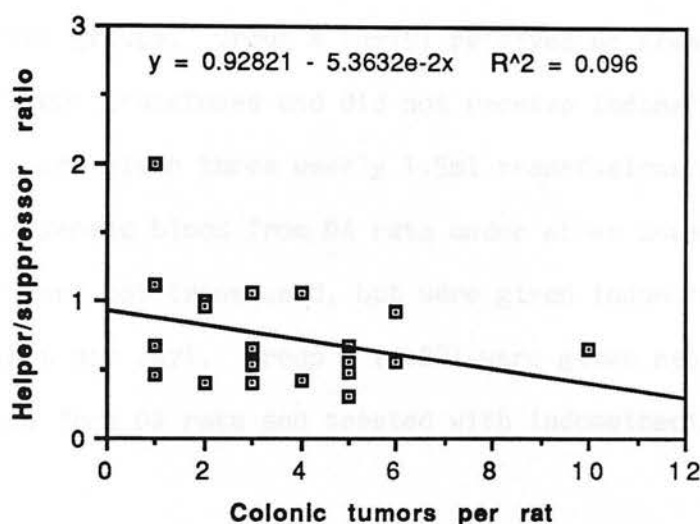


Figure 6.6

Relationship of peripheral blood helper/suppressor (H/S) T lymphocyte ratios to development of dimethylhydrazine colonic carcinogenesis. Correlation of H/S ratio and tumour yield.

6.8. DOES INDOMETHACIN REVERSE BLOOD TRANSFUSION INDUCED  
ENHANCEMENT OF COLONIC CARCINOGENESIS IN RATS?

(Experiment G)

Experimental design

Male Sprague Dawley rats were given 14 weekly injections of dimethyl-hydrazine (16mg/kg). During week 8 they were randomly divided into four groups. Group A (n=19) received no treatment (i.e. they were not transfused and did not receive indomethacin). Group B (n=20) were given three weekly 1.5ml transfusions of heparinised allogeneic blood from DA rats under ether anaesthesia. Group C (n=18) were not transfused, but were given indomethacin (3 mg/kg body weight per day). Group D (n=20) were given heparinised allogeneic blood from DA rats and treated with indomethacin for five weeks.

Seven rats died during the course of the experiment. In Group A, two rats were put down because of respiratory infections; in Group B, one rat died under anaesthesia prior to blood transfusion; in Group C one rat was killed because of a respiratory infection and one was found dead during the 25th week (the cause of death was unclear); in Group D one rat died under anaesthesia prior to transfusion and a second was found dead during week 15 (this rats had pneumonia at post mortem examination).

All surviving rats were killed at 28 weeks. The rats were weighed (Table 6.8.1) and examined for histological evidence of tumours (Tables 6.8.2-3; Figure 6.7). The histological features of every tumour were assessed (Table 6.8.4). The amount of indomethacin given to the rats is given in Table 6.8.5.

TABLE 6.8.1

Dimethylhydrazine carcinogenesis in Sprague Dawley rats treated with allogeneic blood with or without indomethacin: comparison of body weight.

treatment group	blood indomethacin blood and			
	none	alone	alone	indomethacin
	(A)	(B)	(C)	(D)
number of rats	17	19	16	18
weight, g;				
mean (SD)				
1 week 0	270 (67)	297 (89)	281 (80)	291 (86)
2 week 28	659 (161)	670 (183)	669 (176)	656 (179)

There were no significant differences between the groups using student' t test.

TABLE 6.8.2

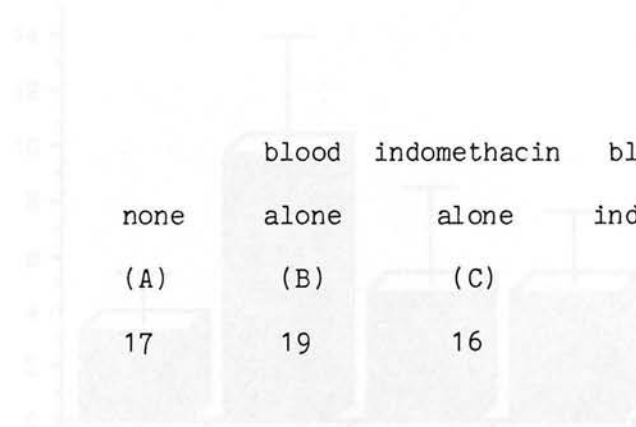
Dimethylhydrazine carcinogenesis in Sprague Dawley rats treated with allogeneic blood with or without indomethacin: comparison of tumour development.

treatment group	blood none (A)	indomethacin alone (B)	blood and indomethacin (C)	blood and indomethacin (D)
number of rats	17	19	16	18
rats with tumours				
colonic	17	19	16	18
small bowel	0	1	0	1
ear	2	0	1	1
metastatic	3	3	2	3

There were no significant differences between the groups using chi square analysis.

TABLE 6.8.3.

Dimethylhydrazine carcinogenesis in Sprague Dawley rats treated with allogeneic blood with or without indomethacin: comparison of colonic tumour development (histologically proven adenoma and carcinoma).



treatment group	none	blood alone	indomethacin alone	blood and indomethacin
	(A)	(B)	(C)	(D)
number of rats	17	19	16	18
colonic tumours	3.29	6.68	4.69	4.67
animal, mean (SD)	(1.76)	(3.97)	(3.46)	(2.57)

A versus B:  $p < 0.02$ , Mann-Whitney test

See Figure 6.7

Effect of blood transfusion (BT), indomethacin alone (Ind) and blood transfusion combined with indomethacin compared with subsequent controls on colonic tumour development in dimethylhydrazine carcinogenesis.  
A versus B:  $P < 0.02$  Mann-Whitney U test.

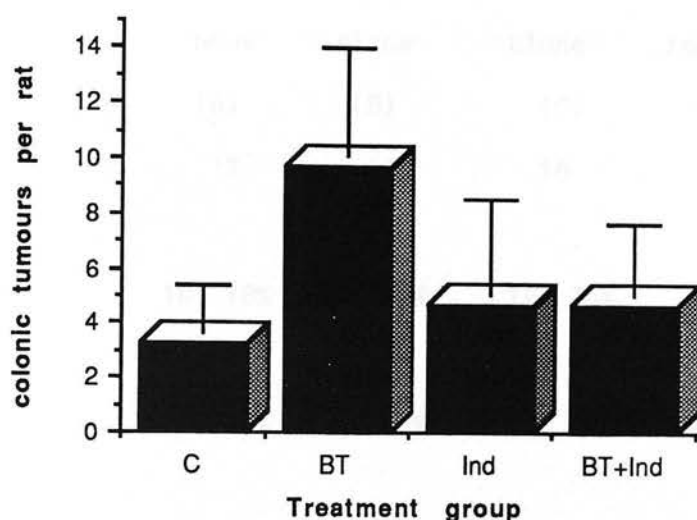


Figure 6.7

Effect of blood transfusion (BT), indomethacin alone (Ind) and blood transfusion combined with indomethacin compared with untreated controls on colonic tumour development in dimethylhydrazine carcinogenesis. Controls versus BT:  $P < 0.02$  Mann Whitney U test.

TABLE 6.8.4.

Dimethylhydrazine carcinogenesis in Sprague Dawley rats treated with allogeneic blood with or without indomethacin.

treatment group		blood indomethacin		blood and	
		none	alone	alone	indomethacin
		(A)	(B)	(C)	(D)
number of rats		17	19	16	18
adenoma; n, %		10, 18%	40, 31%	19, 25%	23, 27%
carcinoma; n, %		46, 82%	87, 69%	56, 75%	61, 73%
tumour histology					
grade	G1	6%	4%	14%	7%
	G2	71%	75%	68%	75%
	G3	23%	21%	18%	18%
mucin production		57%	56%	56%	64%
lymphocyte infiltration		49%	54%	44%	44%
presence of signet cells		13%	6%	11%	6%
invasion					
	pT1	60%	67%	64%	68%
	pT2	40%	33%	36%	32%

There were no significant differences between the four groups, using chi square analysis.

TABLE 6.8.5

Total dose of indomethacin given to experimental subjects.

	indomethacin alone	blood and indomethacin	p*
water drunk per day ml/day; mean (SD)	49.6 (6.64)	45.7 (7.13)	N.S.
body weight kg; mean (SD)	0.560 (0.03)	0.527 (0.01)	N.S.
indomethacin dose mg/kg/day; mean (SD)	1.84 (0.23)	1.75 (0.194)	N.S.

\* t test, p value



6.9. BLOOD TRANSFUSION AND MACROPHAGE PROSTAGLANDIN E2  
PRODUCTION (Experiment H)

Experimental plan

The aim of this experiment was to determine the effects of various blood transfusions on prostaglandin E2 by rat peritoneal macrophages. Lewis male inbred rats (n=104) were divided into eight experimental groups each with 13 rats. On day 0, seven groups were transfused under general anaesthesia, while the eighth group was an untreated control. One of the transfused groups was transfused with anticoagulant solution to control for the effects of anaesthesia and operative procedure. Two groups were transfused with fresh blood, either syngeneic or allogeneic. Two groups were transfused with stored blood, either syngeneic or allogeneic. Two groups were transfused with serum, either syngeneic or allogeneic. On day 4, sterile bovine brain heart infusate was injected into the peritoneal cavity of all rats. On day seven, peritoneal lavage was performed under terminal general anaesthesia. After processing and counting the cell suspensions were cultured in plastic trays. After one hour non-adherent cells were removed and fresh culture media added. After a 20 hour incubation the supernatants were removed and subsequently a radioimmunoassay performed to measure prostaglandin E2 produced by the cells during the 20 hour culture period. A non-specific esterase stain was used to indentify and count the macrophages.

During the experiments seven rats died soon after transfusion, two peritoneal lavage samples were excluded due to contamination with peripheral blood, one of the stored blood units and the relevant spare sample clotted, and in one case the non-specific esterase stain failed in both of the replicate cytocentrifuge preparations. The group distribution of these events can be seen in Table 6.9.1 as there were thirteen rats in each experimental group at the start of the experiment.

Statistical analysis has been performed using a non-parametric method for paired data (Wilcoxon rank sum test). Analysis of the peritoneal cells and the macrophage counts are shown (Table 6.9.1 and Figures 6.8 and 6.9).

Macrophage prostaglandin E2 production during the 20 hour period of culture is shown in Table 6.9.2 and in Figure 6.10.

Table 6.9.1

Effect of transfusion on peritoneal cell counts

Treatment group	n	Total cells mean (SD)	Macrophages mean (SD)
untreated	13	13.5 (7.1)	5.7 (3.25)
anticoagulant	12	13.6 (3.3)	5.6 (2.4)
allogeneic - fresh	12	11.6 (2.1)	4.0 (1.3)
syngeneic - fresh	11	11.9 (3.2)	4.9 (2.2)
allogeneic - stored	10	9.9 (2.3)	3.8 (1.3)
syngeneic - stored	11	10.9 (2.7)	4.2 (1.2)
allogeneic - serum	12	13.4 (6.5)	4.9 (2.7)
syngeneic - serum	12	13.2 (5.6)	4.9 (1.7)

There were no significant differences between the groups:  
Wilcoxon rank sum test.

TABLE 6.9.2.

Effects of transfusions on macrophage PGE2 production

Treatment group	n	PGE2 production/20 hours pg, mean (SD)
untreated	13	3569 (2606)
anticoagulant	12	3863 (1997)
allogeneic - fresh	12	4637 (2803)
syngeneic - fresh	11	4528 (3261)
allogeneic - stored	10	5874 (3726)*
syngeneic - stored	11	6852 (3526)*
allogeneic - serum	12	7956 (6580)*
syngeneic - serum	12	5000 (3557)

\*  $P < 0.01$ : Wilcoxon rank sum test

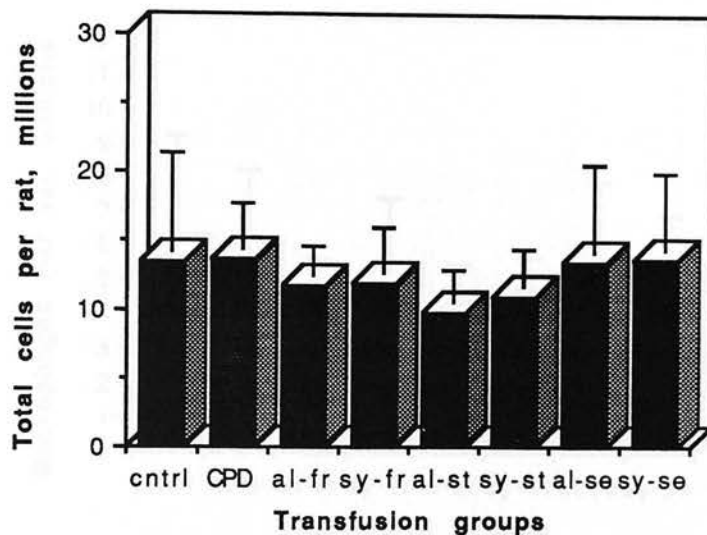


Figure 6.8

Effect of various transfusions on peritoneal cellular response to injection with brain heart infusate. Labels: cntrl = untreated control; CPD = citrate-phosphate-dextrose infusion; al-fr = allogeneic fresh blood; sy-fr = syngeneic fresh blood; al-st = allogeneic stored blood; sy-st = syngeneic stored blood; al-se = allogeneic serum; sy-se = syngeneic serum.

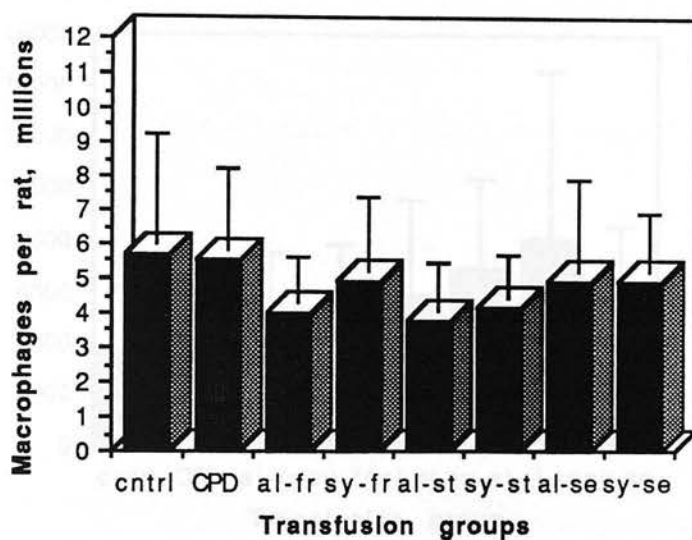


Figure 6.9

Effect of various transfusions on peritoneal macrophage response to injection with brain heart infusate. Labels: cntrl = untreated control; CPD = citrate-phosphate-dextrose infusion; al-fr = allogeneic fresh blood; sy-fr = syngeneic fresh blood; al-st = allogeneic stored blood; sy-st = syngeneic stored blood; al-se = allogeneic serum; sy-se = syngeneic serum.

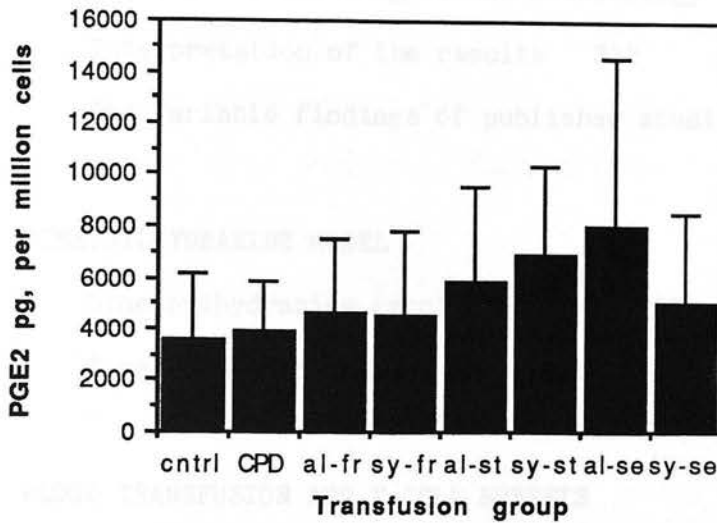


Figure 6.10

Effect of various transfusions on peritoneal macrophage prostaglandin E2 (PGE2) production expressed as PGE2 in picograms produced by one million macrophages during the 20 hour period of culture. Labels: cntrl = untreated control; CPD = citrate-phosphate-dextrose infusion; al-fr = allogeneic fresh blood; sy-fr = syngeneic fresh blood; al-st = allogeneic stored blood\*; sy-st = syngeneic stored blood\*; al-se = allogeneic serum\*; sy-se = syngeneic serum.

\*  $p < 0.01$  when compared with untreated controls: Wilcoxon rank test.

## 7. DISCUSSION

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## 7.1. CLINICAL STUDY

### 7.1.1. Aim of the study

The purpose of this retrospective study was to test the theory that blood transfusion could have a detrimental effect on survival after potentially curative surgery for colorectal cancer.

### 7.1.2. Limitations relating to the methodology

Retrospective studies are known to have many inherent deficiencies which may make it difficult to interpret the perceived differences in survival rates between two treatment groups. These problems are discussed firstly in relation to the current study and secondly in relation to interpretation of the increasing number of reports from other centres.

#### a) Uncontrolled variables

There are many variables which could be controlled for in a randomized prospective trial, but are clearly impossible to control for in a retrospective study:

Previous blood transfusion and pregnancy may be important historical factors which have the potential to affect subsequent immune response to alloantigens. This information

is not obtainable from the cases notes, but in a prospective study the patients could be questioned directly.

Other medical treatment during the follow-up periods may affect the course of the disease in ways as yet unknown. Medications such as aspirin or other non-steroidal anti-inflammatory agents could be prescribed by general practitioners for the relief of symptoms due to metastatic disease or for indications unrelated to colorectal cancer. The patients may use other medications from the field of alternative medicine - evening primrose oil is an example of a compound which may affect the immune response to cancer.

b) Incomplete data is an important factor. In the Methods Section the method of determining blood transfusion status was described and reliance was placed on documentation in the case notes. In this study this documentation was probably reliable because it was an important part of the mechanism of identification used to ensure that mismatched blood was not transfused. However, this methodology does not account for the patient who was cross-matched, but not transfused and who was cross-matched on a second occasion postoperatively and transfused. If by chance the second cross-match form was lost, this patient would have been included, erroneously, in the non-transfused group. There is no reliable information concerning the use of blood products such as platelets, fresh frozen plasma, and plasma protein fractions. These are important because all of these components could influence the immune response and they are more likely to be administered to patients who have had four or

more transfusions of blood.

c) Incomplete follow-up data represents another problem, it is generally thought that if greater than 10% of cases are lost, then the survivorship analysis will be unreliable. However, the log rank methods of survivorship analysis allows for the analysis of studies in which some of the patients have not reached the final (relating to the study) census point. In this study the final census point was five years, but not all patients reached this as the data retrieval was performed at the end of 1984. The patients underwent surgery during the period from January, 1977 to December, 1980. Therefore, a patient who underwent surgery at the end of 1980 could only have been reviewed for 4 years and would be included as lost to follow-up at the 54 month and 60 month census point. Furthermore, after 2 years patients changed from six month reviews to yearly reviews, which means that our December, 1980 patient may not have data for the 42 month and 48 month census points if he or she had had a slightly delayed clinic appointment. The methodology used in this study allows for this eventuality by assuming that this patient's disease behaves in a similar way to the remainder of the study population who continue to be reviewed. To put it another way, it is assumed that the reason for non-review is not related to the patient's state of health or disease progress. Clearly a patient who moves from the district would meet these criteria.

In the present study there was a tendency to an early discharge from follow-up for patients with early tumours. In fact, it has transpired that this was a deliberate (and justifiable) review

policy of one of the consultant surgeons. Furthermore, this surgeon was recognised as having a particular interest in colorectal cancer and he personally performed the definitive resections in 26% of cases reported in this study. Overall, he was responsible for a third of cases (some of these operations were performed by junior surgeons). These patients, who were discharged from follow-up, were usually Dukes stage B with well differentiated tumours and were discharged from the clinic after 18 to 24 months. In constructing the life table analysis these patients would be counted as lost to follow-up and treated accordingly (ie assumed to behave like the rest of the patients remaining in the study). This group of "lost" patients will be weighted because of an excess of patients with a superior prognosis. This will result in apparently poorer survival figures for the whole group at five years. It is recognised that the majority of colorectal cancer recurrence occurs within two years following surgery, but patients will survive beyond this point. This problem of "lost" patients illustrates an important unreliable feature of this retrospective study.

d) The observed effect may be simply co-incidence of phenomena. This is certainly a possibility with this study: the majority (>80%) of the patients had also been included in the prospective multicentre Large Bowel Cancer Project (Cheslyn-Curtis, 1990). This much larger study did not demonstrate a significant blood transfusion effect on survival. Statistically, if 20 centres undertook a similar study, one of them by chance alone could reveal a significant effect of blood transfusion at the  $P=0.05$  level.

e) The effect may be real, but due to other factors which may be associated with the receipt of transfused blood. Many factors will influence the perceived need for blood during the perioperative period. These factors include age, general state of health, cardiovascular ability to withstand blood loss, preoperative anaemia, technical problems in resection, anaesthetic techniques, the skill and experience of the surgeon, and the various doctors' preferred transfusion regime (or lack of regime). Many of these factors will influence cancer prognosis, and many of these factors are difficult to evaluate in retrospective studies.

#### 7.1.3. Interpretation of the results

For the group of patients as a whole (including abdomino-perineal resections), it has been demonstrated that patients who received perioperative blood transfusion had a poorer five year survival rate when compared with non-transfused patients (31% versus 46%). Statistically, this result was of borderline significance using the log rank test. However, using the more sensitive Mantel-Haenszel method this difference is significant ( $p < 0.03$ ). In this group of patients there was a similar trend which suggested that recurrence-free survival rates were inferior in the transfused patients, but this failed to reach statistical significance. This group of 256 patients included 77 (30%) who had abdomino-perineal resections for low rectal cancer. It is well known that cancer at this site has a poorer prognosis and behaves in a different manner than more proximal colorectal tumours. This fact alone could

account for the association between blood transfusion and poor prognosis.

In the present study there was also a higher incidence of annular tumours and staged procedures in the transfused group. It has been shown that survival is poorer after a staged procedure compared with survival after a primary resection for large bowel obstruction caused by cancer (Fielding and Wells, 1974). This increased mortality could be associated with the cumulative cardiovascular risks of anaesthesia in elderly patients. Alternatively, it could be postulated that multiple anaesthesia and surgical trauma cause immunosuppression with a resultant increased risk of sepsis and tumour recurrence. Large bowel obstruction, presumably the sequel of an annular type of tumour, is also known to be associated with a poor prognosis (although an annular tumour, per se, may not have this association). Furthermore, there was a much higher incidence of emergency operations in the transfused group and the commonest indications for emergency surgery are large bowel obstruction and perforation, both of which are associated with a poorer prognosis.

When the patients who had had abdomino-perineal resections were excluded, the association of blood transfusion with poor prognosis was no longer statistically significant. In addition, the higher frequency of tumours adherent to another structure (eg small bowel, uterus, abdominal wall) in the transfused group now reached statistical significance, as did the incidence of pre-operative anaemia. It would appear, therefore, that in this group of patients the survival disadvantage associated with blood transfusion is



likely to be due to differences in the severity of the cancer disease: patients with several variables known to be associated with a poorer prognosis were more likely to be transfused. The receipt of blood transfusion must surely be a marker of disease severity.

Several studies have attempted to account for the influence of known prognostic variables by using sophisticated statistical methods such as the Cox proportional hazards model (Burrows and Tarrter, 1987; Blumberg et al, 1985; Parrott et al, 1986; Creasy et al, 1987). This multivariate analysis demonstrated a significant independent effect of transfusion on cancer recurrence or death. Despite these, it is difficult to counter the argument that perioperative blood transfusion is an indicator of the severity of the cancer disease.

Francis and Judson (1987) found a significantly higher incidence of recurrence in patients who received blood during surgery compared with those who were given blood either before or after surgery. They suggest that factors influencing the need for transfusion during surgery had a greater influence on prognosis than the receipt of blood itself.

This study (like the other retrospective studies) has compared two groups of patients with colorectal cancer. Most studies, including the present, have shown that transfused patients have a poorer prognosis, and some have demonstrated that the difference reaches statistical significance. Before drawing conclusions regarding the possible reasons for this observation, it should be clear that two different groups of patients are being compared.



Although both groups (transfused and non-transfused) may be similar in terms of age, sex, clinical stage, histological grade, and site of the tumour, they must differ in some way to account for the fact that one group was transfused and the other was not. In other words, blood transfusion selects patients who have a poorer prognosis.

When these results are compared with the other retrospective studies of colorectal cancer patients, a further observation may be made. Overall, the survival rates in the present study are markedly inferior to those elsewhere. One explanation for this phenomenon is that patients in this study were drawn from an relatively large geographic area which included rural areas. It is possible that patients with colorectal cancer may have presented at a later stage to the district general hospitals involved in the project. However, there is some pathological evidence to support this. The distance from the patient's home to the hospital was found to be statistically associated with decreased survival in patients with breast cancer (Kieckbusch et al, 1989). However, the hospital concerned was a tertiary referral centre.

#### 7.1.4. The variable findings of published studies

The literature review tabulated the findings of the many studies of colorectal cancer patients. About a third of these concluded that there was a statistically significant relationship between blood transfusion and decreased survival. Another third have indicated a

exert influence such as: anaesthetic techniques and agents, deep venous thrombosis prophylaxis, undescribed differences in the patient populations, intraoperative blood loss, duration of operation, exact time of transfusion in relation to surgery, methods used in exclusion of metastasis may vary (liver ultrasound, CT scan), accuracy of perioperative clinico-pathological staging, nutritional status. Virtually no information is available to enable possible differences in blood banking factors to be considered. Firstly, there may be differences in the blood donor populations; if a virus is responsible for the effects then it is conceivable that these population variations may be transient. The methods of blood processing and storage may vary. During the last fifteen years which cover the time period of the retrospective studies, there has been a trend away from the use of whole blood towards plasma extraction and use of packed cells. The amount of plasma in transfused blood may therefore vary from centre to centre. It will be clear as this discussion proceeds that plasma may be one of the important constituents of blood with regard to immunosuppression.

There may be a threshold level at which a transfusion effect becomes evident. This may not be reached in some transfused patients, perhaps because of variable transfusion policies between centres.

7.2.1 Dimethylhydrazine carcinogenesis (Experiment A)

Rats which received 16 weekly DMH injection had all developed malignancies 36 weeks after the first injection. Of the original 36 rats in this group 18 had died by 30 weeks and at 36 weeks only 9 remained alive. Three of the DMH-treated rats were killed because they had respiratory infections in the early part of the study. None of the 36 rats treated with EDTA injections developed cancer, but one did develop signs of ataxia and upper respiratory tract infection. The untreated control group consisted of 16 rats and none of these developed cancer, but one was killed because of a respiratory infection.

It was surprising to find that at thirty weeks only 50% of the subjects were alive. Beyond this point the surviving rats continued to deteriorate and most of them had developed infected, ulcerating ear tumours. Those animals examined after death at about 30 weeks generally had evidence of metastatic disease and all had obvious multiple bowel tumours. Overall, the mean number of colonic tumours per rat at 36 weeks was 4.78 (SD=3.1)

On reviewing the literature these results can be compared with others. However, this comparison is difficult because of variations in dose, route of administration, total dose of dimethylhydrazine

given, frequency of doses, and strains of experimental rats used (Table 7.2.1).

TABLE 7.2.1. DMH rat models of colonic carcinogenesis

Author, year	Strain	Sex	Dose	Total	Route	End point	Tumours/rat mean
			DMH,mg	dose,mg			
Ward, 1974	Fischer	M	26.6	532	sc	30	3.9
						38	4.7
Garmaise, 1974	SD	M	30	300	o	22	1.7
Reddy, 1974	Fischer	M	10	200	sc	20	0.8
Rogers, 1975	SD	M	30	150	o	30	20% dead
						40	50% dead
						45	90% dead
Maskens, 1976	BDIX	M	20	460	sc	36	8.0
Barkla, 1977	SD	M	21	420	sc	30	6.0
Filipe, 1975	Wistar	M	20	580	sc	26	4.0
Reddy, 1976	Fischer	M	10	200	sc	30	0.25
This study	SD	M	16	256	sc	36	4.8
	SD	M	16	224	sc	28	3.3

Data from Experiment A and from Experiment G (untreated controls) refers to rats surviving at the end point.

What are the possible explanations as to why the present protocol resulted in apparently high mortality?

It is possible that the present experimental rats developed more extensive metastatic disease which accounted for their early demise. This is difficult to assess. Of the 13 rats killed deliberately during the experiment, only two had macroscopic evidence of metastatic disease. Of the 14 rats which died spontaneously, only 4 had evidence of metastatic disease. Five of the nine rats reaching 36 weeks had various distant metastases. It would seem unlikely that metastatic disease caused the high mortality rate.

On review of the experimental records it was noted that four additional rats in the DMH group had had signs of respiratory infection, but not severe enough to warrant killing them. From reviewing the data from other papers it would seem that the degree of carcinogenesis in this study was average. However, the mortality rate in this study is undoubtedly high in comparison. It is likely that the high incidence of respiratory infections may have accounted for this high mortality. Respiratory infection was not a problem in the other carcinogenic experiments (Experiment G and Appendix 2). Neither of these experiments was associated with a high mortality rate, although the experiments were terminated at 28 and 30 weeks respectively.

It will be seen that 56% of the group of DMH-treated rats developed ear tumours. These were squamous cell carcinomas of the external auditory canal. The observed incidence is higher than those previously reported (Druckrey, 1970; Bansal et al, 1978; Martin et al, 1973). However, in a small series reported by Cruse et al (1978) 4/9 female Wistar rats treated with dimethylhydrazine and *Corynebacterium parvum* developed ear tumours. An incidence of 36% of rats with ear tumours was found by Ward (Ward et al, 1974). Reddy et al found that in rats treated with 20% corn oil, 59% developed ear tumours compared with 15% in rats fed a standard diet. Several other reports of experimental carcinogenesis do not mention ear tumours - this suggests that it was not a problem. The high incidence of ear tumours is important because of the natural course of the disease. Eight of the 13 rats killed prematurely because of the presence of ulcerating and infected ear tumours.

During the course of the experiment three rats injected with DMH developed skin necrosis at the injection site. In subsequent experiments injections were given in alternate flanks. This area of dermal necrosis measured about 1cm in diameter. It is feasible to suggest that this dermal necrosis may have caused a degree of immunosuppression in a manner similar to a burn wound (Wood et al, 1987). This would render the animals more susceptible to infection or the carcinogenic process. However, the small number of rats involved would make this an insignificant factor.

It is known that a diet with a high fat content may enhance carcinogenesis (Bansal et al, 1978; Sakaguchi et al, 1984; Reddy,

1976). The standard rat diet used in the present study has an average fat content (3.5%) and this level has not been reported to increase carcinogenesis.

Various other factors are known to enhance carcinogenesis such as drugs which may alter the bowel flora (A-Kareem et al, 1984; Nigro et al, 1973), protamine (Phillips et al, 1984), anaesthesia and surgery (Weese et al, 1986), pregnancy (Sjogren, 1980). However, none of these factors seem to be relevant in this experiment.

It is possible that the experimental rats were immunosuppressed due to a cause unknown and this caused the high incidence of respiratory infections and ear tumours, and ultimately the high mortality. The relationship of this theory to the observed T lymphocyte subset analysis is discussed later. It is also possible that any immunosuppression was a direct consequence of the unidentified pathogen which caused the respiratory infection or a direct consequence of the malignant ear and bowel disease.

In summary, it is suggested that the unexpected high mortality observed in this experiment was due, firstly, to premature culling of rats with respiratory infection. Other rats in the carcinogenesis group were also noted to have had signs of respiratory infection which may have contributed to the number of spontaneous deaths. Secondly, the high incidence of ear tumours resulted in additional rats being culled prematurely because of infective complications.

7.2.2 T cell subsets and the cancer model

T cell subsets in normal rats

Throughout the early experiments when T lymphocyte subsets were enumerated, the results showed consistently low helper/suppressor ratios in the various groups of normal rats which were untreated controls (Table 7.2.1).

TABLE 7.2.1. Helper/suppressor ratios in normal rats

Experiment	age of rat (weeks)	helper/suppressor ratio*
B	16	0.74
	40	0.63
	40	0.57
C	12	0.88
D	14	1.04
	16	0.75
	36	0.62
	36	0.61

\* helper/suppressor ratio = %W3/35 +ve cells / %OX8+ve cells



What is the normal helper/suppressor ratio for Sprague Dawley rats?

A literature search revealed little information or data concerning helper/suppressor ratios in normal rats. However, although there appears to be marked strain variation a helper/suppressor ratio of less than one must be considered to be abnormal. Naturally lymphopenic BB rats (non-diabetic) were found to have a mean helper/suppressor ratio of 0.86, while normal Wistar Furth rats had a ratio of 1.31 (Elder et al, 1983). Another report stated that normal Wistar Furth rats had a helper/suppressor ratio of 1.3, but this became less as the rats aged more than one year (Ross et al, 1984b).

Young WAG rats had a mean ratio of 1.76 in another series of experiments (Lennard et al, 1986). Normal Brown Norway rats have a ratio of 3.0 (Fernandez-Cruz et al, 1982)

#### **Causes of low helper/suppressor ratios**

The effect is unlikely to be spurious because similar results were observed in several groups of normal rats (Table 7.2.1).

The methodology used may have been deficient in some way. There is a predominance of cells labelled with the OX8 monoclonal antibody.

At first it was suggested that the W3/35 and OX8 monoclonal

antibodies had been confused during aliquoting of batches. However, this was excluded by cross-checking labelling with the original batches with new batches (data not shown).

Non-specific binding was thought to be a problem in pilot experiments which used a monoclonal antibody preparation derived from ascitic fluid. When a supernatant preparation was introduced this reduced the amount of non-specific binding as seen on the FACS machine, but did not obviously affect cell counts.

The observed helper/suppressor ratios may have been real.

Functional studies show that OX8 labels cells which have suppressor activity (Mason et al, 1981b) and also cytotoxic T cell precursors and effector cells (Dallman et al, 1982). OX8 positive cells probably include natural killer cells. Predominance of OX8 positive cells is usually correlated with immunosuppression, but clearly there are other cytotoxic cells present. The frequency of rats with respiratory infections during the period of these early experiments has been described already. It is possible that a virus infection may have affected many of the rats and caused immunosuppression which in turn caused the high mortality. Such an immunosuppressive agent may well have caused alterations in the lymphocyte sub-populations.

However, if the rats with low helper/suppressor ratios are immunosuppressed, then one might expect dimethylhydrazine carcinogenesis to be enhanced in this group. There was no

correlation between helper/suppressor ratios and the number of colonic tumours developed in rats treated with DMH (Figure 6.6).

Turning to T lymphocyte subsets in humans, there appears to be a marked variation as seen between various strains of rat. In humans T cell CD4 and CD8 subset ratio changes have been shown to correlate with progression of human immunodeficiency virus-related disease (Macy et al, 1988). A large cohort of healthy people was studied to establish a normal range of CD4/CD8 ratios. The ratios of 1.00 to 3.15 were established as the 5th and 95th percentile. This also meant that 5% of normal healthy people had "abnormally low" CD4/CD8 ratios. Clearly, to establish the relevance of the low helper/suppressor ratios seen in the current experimental rats, further studies should be undertaken to compare peripheral blood lymphocyte subsets in a range of rat strains.

#### Experiment B

The purpose of Experiment B was to determine the distribution of peripheral T lymphocyte subsets in normal Sprague Dawley rats of varying ages. There were no differences in the proportions of labelled cells making up the subsets when 16 week old rats were compared with 40 week old rats. It is admitted that these ages probably correspond to pubertal and young adult developmental stages in the rat rather than adult and geriatric. There are no reports concerning peripheral T lymphocyte subset changes associated with age in rats. However, one paper reports that in Brown Norway rats the percentage of lymphocytes (isolated from the spleen and lymph nodes) labelled with Thy-1 monoclonal antibody was smaller in old

rats compared with young rats (Gilman et al, 1981). The authors found no differences regarding cells labelled with W3/35 antibodies. They suggested that homeostatic functions controlling the immune system may not be functioning in aged rats.

### Experiment C

The purpose of Experiment C was to determine the distribution of T lymphocyte subsets in the various groups of Sprague Dawley rats in the carcinogenesis model. Of the original 36 rats in this group 18 had died by 30 weeks and at 36 weeks only 9 remained for analysis of lymphocyte subsets.

The rats with advanced cancer had a larger proportion of W3/25 labelled T lymphocytes (helper) compared with age matched normal rats. The rats with cancer also had fewer OX 8 labelled T lymphocytes (suppressor/cytotoxic) compared with age-matched rats treated with EDTA. Compared with normal rats, EDTA-treated rats had a greater proportion of OX 8 (suppressor/cytotoxic) cells.

The effect of DMH carcinogenesis on T lymphocyte subsets has been studied in Wistar Furth rats (Ross et al, 1984). It was found that T lymphocyte subpopulations did not change until tumours had developed and even then differences were found only at 28 weeks after the first injection. Compared with six age-matched controls, six DMH-treated rats possessed a larger proportion of OX 8 (suppressor/cytotoxic) labelled lymphocytes and a smaller proportion of W3/25 (helper) labelled cells. The helper/suppressor cell ratios

were compared and the difference between the mean ratios was significant. The results of the present study do not agree with this and the most obvious reason may have been the presence of secondary bacterial infections in the ulcerated ear tumours.

The significance of the changes associated with EDTA injections is difficult to explain. The observation may simply be coincidental ( $P=0.05$ ). None of the rats treated with EDTA developed skin necrosis at the injection sites and only one of these rats developed sign of respiratory tract infections and was culled prematurely. The same infective problems which affected DMH-treated rats also affected the EDTA-treated rats, but to a lesser extent. The increase in OX 8 cells may reflect immunosuppression due to the repeated trauma of the injections. Alternatively it could be due to the effect of EDTA itself. There is no literature to support a direct toxic effect on stem cells or lymphocytes.

Infection via a contaminated EDTA stock solution is a possibility which cannot be excluded. Bacterial infection is less likely because the stock solution was sterilized by filtration prior to aliquoting and freezing, although no bacteriological cultures were performed on either the EDTA or DMH/EDTA solutions.

A metabolic effect of EDTA is hypocalcaemia as EDTA chelates calcium ions (and probably other ions of similar valency). A series of sixteen weekly injections could, conceivably, cause chronic hypocalcaemia. EDTA has been used to treat Paget's disease of the

bone and it is recommended that calcium supplements are given concurrently. There are no reports of immunological side effects in vivo associated with this treatment.

Schnaper studied the effects of EDTA on the suppressive of soluble immune response suppressor (SIRS) on murine plaque-forming cells in vitro (Schnaper, 1989). SIRS is an immunosuppressive protein which requires activation to SIRSox by peroxide. Purified hybridoma-derived SIRS was treated with chelating agents which included EDTA. Suppressing activity was reconstituted by subsequent treatment of SIRS with  $\text{FeSO}_4$ ,  $\text{NiSO}_4$ , or  $\text{MgSO}_4$ , but not by  $\text{FeCl}_3$ ,  $\text{MnSO}_4$ ,  $\text{CuSO}_4$ ,  $\text{ZnSO}_4$ ,  $\text{CaCl}_2$  or  $\text{CrCl}_3$ . These results indicated that SIRS requires a divalent metal ion for activity. The effect of EDTA treatment in the rat model was associated with a change of the helper/suppressor ratio suggestive of immunosuppression and this clearly cannot be related to SIRS-related effect unless it affected some of these trace elements.

It is possible to form a hypothesis with regard to the possible role of hypocalcaemia in altered T lymphocyte subsets. An increase in the concentration of cytoplasmic free  $\text{Ca}^{2+}$  and the activation of protein kinase C appear to play prominent role in initiating lymphocyte responses (Weiss et al, 1986; Cambier et al, 1987). It is thought that these two intracellular signals are the result of receptor-induced hydrolysis of membrane polyphosphoinositides (PPI) (Kuno et al, 1986). The breakdown of PPI generates two second messengers - inositol 1,4,5 triphosphate ( $\text{IP}_3$ ) which releases  $\text{Ca}^{2+}$  from an intracellular source. Secondly, diacylglycerol (DG) is

produced which activates protein kinase C (Kuno et al, 1986). However, it is thought that the second mechanism is associated with an influx of extra-cellular  $Ca^{2+}$  possibly through a plasma membrane channel (Kuno et al, 1987). Without going to deeply into this topic, it is possible that depletion of body calcium by the chelating action of EDTA could interfere in this immunoregulatory system and result in change in T lymphocyte populations.



### 7.3. BLOOD TRANSFUSION AND T CELL SUBSETS

#### 7.3.1 Transfusion in normal young rats

In Experiments D1 and D2, normal 16 week old rats transfused with blood or saline showed a decrease in the proportion of T lymphocytes in peripheral blood. In these younger rats there was no effect on the helper or suppressor/cytotoxic populations of peripheral lymphocytes. It will be noted that four different statistical methods have been used to analyse the the two groups of results. These have clearly given rise to varying degrees of significance. The rationale for using the t test in this situation has already been discussed.

#### 7.3.2 Transfusion in normal aged rats

In the older rats (Experiments D3 and D4) the total T lymphocyte proportions did not change following transfusion, whether it was blood or saline. In the older rats, allogeneic blood transfusion was associated with an increase in the OX 8 T lymphocyte proportion. In older rats transfused with saline only a small insignificant increase in OX 8 cells was seen, but the W3/25 (helper) lymphocytes appeared to increase in this group.

Lennard et al (1986) reported that in WAG rats, allogeneic blood transfusions caused a "down regulation of the immune response by



altering the ratio of the immunoregulatory lymphocyte subpopulations". The monoclonal antibodies used were W3/13, W3/25 and OX 8 and the subsets were counted using flow cytometry. The present series of experiments involved rats of different strains and these results suggest that T lymphocyte helper and suppressor subpopulations in normal young rats are not influenced by allogeneic blood transfusion. This would appear to contradict the findings of Lennard et al. However, it has been demonstrated that in older rats allogeneic blood transfusion may cause T lymphocyte population changes which are indicative of an immunosuppressive state. It has been suggested that blood transfusion may have a detrimental effect in cancer patients. It is possible, therefore, that this effect may be greater in elderly patients with cancer than in younger patients. It should be interesting to investigate this theory further on a clinical basis.

### 7.3.3 Transfusion in dimethylhydrazine-treated rats

The effects of transfusion with allogeneic blood and saline were assessed in Sprague Dawley rats receiving dimethylhydrazine injections (Experiments E1 and E2). It will be recalled that 16 week old rats transfused with blood or saline showed a decrease in the total T lymphocyte count following transfusion, but no significant change in T cell subsets. Age matched rats receiving weekly injections of dimethylhydrazine were transfused concurrently with the rats in Experiments D1 and D2. Rats undergoing chemical carcinogenesis did not demonstrate a drop in the proportion of T

lymphocytes in peripheral blood. When transfused with blood there was no change in the proportions of W3/25 and OX 8 cells in the peripheral blood. However, when these rats were transfused with saline, this resulted in a decrease in the proportion of lymphocytes labelled with W3/25 monoclonal antibody. In this group of rats there was a post-transfusion decrease in W3/13 labelled T lymphocytes. Although this was not statistically significant, it may have accounted for the observed post-transfusion (saline) decrease in helper cells.

#### 7.4            INDOMETHACIN, BLOOD TRANSFUSION AND COLONIC CARCINOGENESIS

##### Experiment F

The aim of this experiment was to investigate the possibility that indomethacin, a prostaglandin synthetase inhibitor, might influence the enhancing effect of allogeneic blood transfusion on dimethylhydrazine colonic carcinogenesis.

##### 7.4.1        Experimental design

Dimethylhydrazine was administered as weekly subcutaneous injections at a dose of 16 mg/kg body weight for a period of 14 weeks. In the previous experiments dimethylhydrazine was given for 16 weeks. The total dosage was decreased in the present experiment in order to reduce the incidence of advanced cancer which tended to cloud the overall results in the other series of experiments (Ross et al, 1988: Appendix 1). Rats were also killed earlier at 28 weeks. This regime appeared to be effective. Noticeably, there were fewer rats which developed ear tumours and there was a smaller incidence of rats with small bowel tumours and metastatic disease.

If further experiments were planned, it is suggested that a suitable regime would be 14 weekly injections of dimethylhydrazine (16 mg/kg body weight) with assessment by post mortem examination at 24 weeks.

This experiment included four experimental groups of rats, all of which were given the carcinogen. One group received no additional treatment. Two groups of rats received allogeneic blood transfusion and one of these was treated with oral indomethacin before, during and after transfusion. The fourth group, not transfused, was treated with indomethacin for an identical period.

Ideally, two more groups could have been added. Both these would be transfused with saline and one would also receive indomethacin. These groups would have controlled for the possible effect of repeated anaesthesia and surgical trauma required to perform the transfusions. In the present experiment the group transfused with blood developed more tumours than the control group (6.7 versus 3.3;  $P < 0.02$ ). Previous experiments showed that up to 34 weeks after the start of tumour induction, the number of tumours per rat increased with time (Ross et al, 1989: Appendix 1). The slightly smaller number of tumours observed in the present experiment is compatible with this observation. It is also likely that an additional group transfused with saline would have a similar degree of carcinogenesis to the untreated control group.

#### 7.4.2. Microscopic tumour development

This experiment confirms that allogeneic blood transfusion enhances colonic carcinogenesis to a degree which is similar to that observed in previous work (Ross et al, 1989: Appendix 1). Administration of indomethacin at a dose of about 1.8 mg/kg body weight per day for a

a period of four weeks does not influence carcinogenesis. some evidence, which has been reviewed, which suggests that long-term treatment with indomethacin may retard dimethylhydrazine carcinogenesis in rats (Pollard et al, 1977,1978 and 1983).

Administration of indomethacin during the period of blood transfusion appears to reverse the enhancing effect of transfusion on carcinogenesis. In transfused rats given indomethacin, the number of microscopic tumours was the same as in the group which received indomethacin alone.

The incidences of ear tumours, small bowel tumours and metastatic disease were similar in all four groups.

Indomethacin is a prostaglandin synthetase inhibitor. It would be reasonable to suggest that it is possible that the blood transfusion effect seen in this model may be mediated by a mechanism involving prostaglandins.

In experimental models, allogeneic blood transfusion has been shown to enhance graft survival and these effects can be negated by administration of indomethacin (Shelby et al, 1987). It is suggested that a prostaglandin-mediated immunosuppressive effect of blood transfusion may be present in the present cancer model (which does not involve tumour transplantation) and that this effect may share similar or identical mechanisms as the known beneficial effect of blood transfusion on experimental graft survival.

## 7.6. BLOOD TRANSFUSION AND MACROPHAGE PROSTAGLANDINS

### 7.6.1. Blood transfusion and macrophage arachidonic acid metabolism (Experiment G)

The aim of this experiment was to determine the effect of allogeneic blood transfusion on macrophage synthesis of arachidonic acid metabolites. Secondly, the experiment was designed to test the hypothesis that storage conditions of transfused blood might influence the effects of macrophage PGE2 production.

The experimental design was based on that described by Waymack et al who had shown that allogeneic blood transfusion resulted in increased PGE2 production by rat peritoneal macrophages (Waymack et al, 1987). A pilot study had been performed by the author previously and there were major differences between the results of this pilot study and those of Waymack (Beavis, Ross et al, 1989: Appendix 2). This pilot study did not demonstrate an increased PGE2 production by rat peritoneal macrophages associated with allogeneic blood transfusion. Blood transfusion in the pilot series was 1.5 ml of blood instead of 1 ml and the rat strains differed. The pilot series used DA rats as blood donors and Sprague Dawley rats as recipients. In the Waymack series Lewis rats were used as recipients and Buffalo rats or A'Sogaloff Cancer Institute (ACI) rats were used as blood donors. Waymack used blood which had been mixed with citrate-phosphate-dextrose anticoagulant solution and

stored the blood at 4°C for 24 hours prior to transfusion. In the pilot series fresh heparinized blood was used.

In previous experiments it was observed that removal of the medium from the plastic wells and repeated washing to remove non-adherent cells after one hour of incubation resulted in a high degree of cell loss. It has been shown that the amount of prostaglandin E2 produced by non-adherent cells was minimal (see Methods Section). Therefore, in the current series of experiments this washing step was omitted.

Waymack had cultured the elicited macrophages for a period of 24 hours after the first hour prior to removal and replacement of medium. At 24 hours his control (3mL intravenous bolus infusion of Ringer's lactate solution) rat macrophages produced 740 pg PGE2 per  $10^6$  macrophages. In the present series it was found that at 20 hours the control (NaCl/CDP) Lewis peritoneal macrophages produced 3863 pg PGE2 per  $10^6$  cells. This obvious difference could be explained by the disparity in the number of macrophages retrieved from the peritoneal lavage fluid. In this experiment  $13.6 \times 10^6$  cells were retrieved of which  $5.6 \times 10^6$  were macrophages as identified by the non-specific esterase stain technique. Waymack retrieved a mean of  $9.9 \times 10^6$  macrophages per rat - he does not state the total cells retrieved, but states that macrophages were counted using an automated cell counter. This morphological method of enumerating macrophages may be less accurate than histocytochemical analysis.

The present results show that PGE2 production is increased by all categories of transfusion, but only reaching significance in the allogeneic stored and serum and syngeneic stored groups. The strains of rat used in the present series (Lewis RT1<sup>l</sup> and DA RT1<sup>a</sup>) differed from those of Waymack (Lewis RT1<sup>l</sup> and ACI RT1<sup>a</sup>), but were similar in terms of rat histocompatibility status. In both series the rats used were inbred.

As a short period (up to 90 minutes) elapsed before actual transfusion of "fresh" blood the same mechanism at play in the stored blood may have been responsible for the obvious (but not statistically significant) rise in PGE2 production seen in both groups transfused with fresh blood compared with both control groups. It would appear therefore that the effect of blood transfusion may be due to storage conditions of blood and that this effect is independent of the genetic properties of the transfused blood. Indeed, Waymack found a 40% increase in PGE2 synthesis after transfusion with syngeneic Lewis blood, but this did not reach statistical significance. However, they were able to demonstrate significant increases after transfusion with allogeneic blood. Transfusion with blood from Buffalo rats resulted in similar PGE2 synthesis to that found after transfusion with ACI rat blood. This finding does not suggest a variation in results which depends on the strain of rat used as donors, further supporting the postulated role of non-genetic factors in this model.

In the present model allogeneic serum had a much greater effect than syngeneic serum. This is difficult to explain because serum does



not contain cells bearing histocompatibility antigens, although it is not known whether histocompatibility antigens are released into serum as blood clots.

#### Known immunological effects of stored blood.

There are very few reports which concern the immunological effects of relating to the storage conditions of transfused blood.

Transfused whole blood is defined as: blood collected into an anticoagulant/preservative solution and not processed in any way.

It contains all the cellular and plasma constituents of blood, subject to changes on storage, with the exception of ionized calcium which is precipitated by citric acid in citrate-containing anticoagulants. Blood is stored at 4 to 8°C and the following changes are known to take place during storage:

Red cells - rapidly lose ATP and 2,3-DPG on storage. ATP loss can be slowed by the addition of adenine to CPD solution. The clinical significance of 2,3-DPG loss is uncertain at present, but it is reversible.

White cells - granulocytes begin to lose their phagocytic and bactericidal properties within 4-6 hours and are non-functional after 24 hours storage. However, they do not appear to lose their antigenic properties.

Platelets - lose their haemostatic properties within 48 hours.

Coagulation factors V and VIII (also XI) have less than 50% of their initial activity within 48-72 hours.

Biochemical changes in stored blood include a fall in sodium concentration, a drop in pH, a rise in potassium concentration, a fall in glucose levels and a rise in the concentration of inorganic phosphate.

Vliet et al studied the the in vitro suppressive effects of banked blood (Vliet et al, 1989). Recalcified and heat-treated plasma and supernatants of blood were added to mixed lymphocyte cultures composed of cells from normal subjects. They found that plasma from citrate-phosphate-dextrose solutions with adenine had the most marked inhibitory effects on the proliferative response of normal lymphocytes. Further experiments showed that this effect was not due to the anticoagulant/preservative solutions. These effects were seen from the first day to 28 days of storage. There were no changes in the lymphocyte subsets associated with storage although others have reported technical problems relating to the effects of low temperatures on lymphocyte cell membranes and to flow cytometric considerations (Van Lambalgen et al, 1985; Nicholson et al, 1984).

It has also been shown in a rat model that the killing ability of natural killer cells was markedly inhibited by citrated rat plasma (McCulloch et al, 1988). This effect was similar in both allogeneic and syngeneic preparations. Conversely, 10% rat serum had no effect. The findings of Hormini et al, have been discussed in the Review section: they found that amongst various transfusions plasma

had the greatest enhancing effect on growth of transplanted tumours in mice (Hormini et al, 1983).

The study by Carty et al has been mentioned previously: they studied the effect of transfusions of syngeneic blood, which had been stored for three weeks prior to use, on azoxymethane colonic carcinogenesis in Sprague Dawley rats, but found no effect (Carty et al, 1989). However, their paper does not describe the type or amount of anti-coagulant/preservative used and this limits interpretation of their results in the current context.

## 7.7. BLOOD TRANSFUSION AND CANCER

### - POSSIBLE EXPLANATIONS AND MECHANISMS

Several authors have reviewed the topic of blood transfusion and colonic cancer, but there is no clear consensus regarding possible mechanisms. Fielding (1985) drew attention to the work of Medawar who demonstrated that blood transfusion is an immunostimulant in a "normal" host and that forty years later immunologists cannot readily explain the effects of blood transfusion in renal transplant recipients. George and Morello (1986) concluded that while studying the effects of blood transfusion the influence of the underlying disease state must be considered. They also pointed out that in experimental models blood transfusion has been shown to augment or increase tumour growth and that these differing effects may be due to use of differing strains of host and donor animals. Waymack and Alexander (1986) felt that the effects of blood transfusion were more likely to be due exposure to foreign antigen than due to a non-specific effect.

#### 7.7.1. Blood transfusion is an index of advanced disease

Many of the retrospective clinical studies reviewed previously have indicated that patients who received blood transfusion may have had more advanced cancer. For example, in the study reported in this thesis transfused patients had a higher incidence of preoperative anaemia and a higher incidence of tumours adherent to other

structures. These are not major prognostic variables in relatively small studies. In larger series authors have attempted to take account of bias introduced by confounding prognostic variables. Parrott et al (1986) found a higher incidence of preoperative anaemia and longer operations in colorectal cancer patients who were transfused perioperatively. However, they were able to adjust for this and still demonstrate poorer survival in transfused patients.

Tartter prospectively studied cell-mediated immunity in patients about to undergo resection of colorectal cancers (Tartter and Martinelli, 1987). It was found that the preoperative blood count, T lymphocyte subset numbers and percentages, and natural killer cell cytotoxicity were lower in patients who were subsequently transfused, although the differences were not statistically significant. If these findings were confirmed in a larger study, then this would support the theory that blood transfusion is a marker of disease severity. Therefore, the argument that transfused patients will receive more blood because of the site and intrinsic nature of their tumour and that they have a poorer prognosis because of their tumour will remain tenable until prospective studies prove otherwise.

#### 7.7.2. Comparison with transplantation

At present, the exact mechanism responsible for the "transfusion effect", which has been observed in renal transplant recipients, has yet to be defined. It is therefore premature to suggest that the

same mechanism or mechanisms are responsible for fact that transfused cancer patients have a poorer prognosis than non-transfused patients.

There are major differences between colon cancer patients and renal transplant patients. Although both groups of patients are to some extent immunosuppressed due to their disease, transplant patients are pharmacologically immunosuppressed with steroids, azathioprine and cyclosporin. Cyclosporin has been introduced after the effects of blood transfusion were recognised and it is likely that the enhanced graft survival associated with cyclosporin will diminish the overall beneficial effect of blood transfusion in transplants.

Secondly, transplant patients will be transfused prior to transplantation (i.e. before they are exposed to the antigenic stimulus). Both groups of patients can be transfused postoperatively and there is evidence, which has been reviewed, to suggest that in transplant recipients, postoperative or perioperative transfusion will also enhance survival.

If the transplant effect operates through a nonspecific mechanism mediating immunosuppression, then it would be reasonable to assume that such an effect could act in cancer patients.

If the transplant transfusion effect is specific and depends on histocompatibility antigens, then it is more difficult to envisage a similar mechanism for colonic cancer patients. Transplanted kidneys are recognised and are rejected because they express foreign

histocompatibility antigens. Previous exposure to foreign antigens shared with the transplanted tissue can result in tolerance and enhanced graft survival. This has been demonstrated experimentally (Hutchinson and Morris, 1986). For this mechanism to work in cancer patients, there must be foreign histocompatibility antigens present on cancer cells to which the host immune response is modulated by previous or subsequent exposure to similar foreign antigen in transfused blood. The tumour cells have been presumably present for some time within the lesion before surgery, but we know that tumour cells are dispersed at the time of tumour resection.

#### 7.7.3. Tumour emboli and potential metastases

Recurrence can develop locally or at distant sites. Suture line recurrence is a problem and it has been demonstrated that viable exfoliated tumour cells are present at the anastomotic site and these must be considered as a possible cause of local recurrence assuming that the tumour itself has been completely excised (Umpleby et al, 1984). Tumours are also dispersed via the blood stream. Engell (1955 and 1957), working in Copenhagen has extensively investigated this topic. He obtained venous blood samples during surgery from patients undergoing cancer resections. Out of 76 patients with rectal cancer, tumour cells were identified in venous blood in 41 cases. In cancer of the colon 22/31 patients had cancer cells present in venous blood. These findings did not appear to have a prognostic significance and cancer cells were found with a similar frequency in patients who survived without recurrence

as in those who developed recurrence. Interestingly, he could not demonstrate an increase in the number of circulating tumour cells following operative manipulation. The presence of tumour cells in blood during resection was found to be related mainly to histological differentiation and to a lesser degree with local extension. Salsbury et al (1965) found that the incidence of malignant cells in the common iliac vein during resection of the colon and rectum was much greater after high ligation of the inferior mesenteric vessels than before. This suggested that there could be a retrograde flow of venous blood into the systemic circulation via the middle rectal and pelvic veins even after ligation of the main venous drainage.

Jeekel (1986) reported the early results of a clinical trial to assess the effects of a "no-touch" technique of colonic resection for carcinoma. The essence of this method was early ligation of the vascular pedicle and mesenteric structures before mobilisation of the colonic segment. In patients with sigmoid colon cancer treated conventionally, 20% developed liver metastases compared with only 7% of patients in the "no-touch" technique.

In summary, there is evidence that with or without operative manipulation tumour cells are released into the circulation. These cells may form tumour emboli which could develop into metastatic deposits. It is these cells which may represent a source of antigenic stimulus during the perioperative period. The type of antigenic stimulus will clearly depend on the antigens expressed on the surface of the cancer cells.



#### 7.7.4. Cytokines as mediators of the transfusion effect

##### Prostaglandin E2

Other work has indicated that prostaglandin E2 is involved in immunosuppression caused by blood transfusion and these have been discussed in the Review Section, but they are summarised here.

Prostaglandin E levels have been found to be raised in dialysis patients who received multiple transfusions (Jackson et al, 1985; Roy et al, 1985; Lenhard et al, 1985). Injection of sheep red blood cells into mice resulted in increased immunosuppressive PGE production by splenic macrophages (Webb and Osheroff, 1976). More recently, Shelby has shown that indomethacin blocks the blood transfusion-induced immunosuppression associated with a prolonged graft survival in a rat heart transplant model (Shelby et al, 1987). It was also shown that anti-PGE antibody blocked transfusion associated immunosuppression by neutralising endogenous PGE2 in the experimental animals.

Waymack's finding that blood transfusion increases PGE2 production by macrophages has already been discussed.

## Interleukin 2

There is now more recent evidence that the cytokine interleukin 2 (IL-2) may be involved in transfusion-mediated immunosuppression. To determine the effect of blood transfusion on IL-1 and IL-2 production and IL-2 receptor expression by mouse splenic cells, Wood et al studied cells which had been characterised as suppressive in terms of decreased cell-mediated lymphocytotoxicity and suppression of blastogenesis in mixed lymphocyte culture (Wood et al, 1988). The production of IL-1 by transfused mice was normal, but the production of IL-2 was significantly suppressed when measured at 2 and 16 days after transfusion. IL-2 receptor expression was normal after transfusion. Furthermore, the addition of IL-2, but not IL-1, to cell-mediated lymphocytotoxic assays restored the diminished response to normal levels. Further experiments suggested that the suppressor cells derived from the spleen were macrophages and not T lymphocytes.

Another study tested the effect of blood transfusion on antigen presentation function of peritoneal macrophages and IL-2 production by splenocytes in mice (Stephan et al, 1988). Antigen presentation function was tested by using D10.G4.1 T lymphocyte clone and conalbumin - a specific antigen for for these cells - and measuring D10.G4.1 proliferation induced by processing of the antigen by peritoneal macrophages. In this model, blood transfusion had no effect on the antigen processing and presentation functions of macrophages. However, the splenic cell production of IL-2 was significantly reduced after only one transfusion.

In a rat renal transplant model in which allogeneic transfusion enhanced graft survival, Dallman et al found that in vivo administration of recombinant IL-2 caused acute rejection in transfused transplant recipients (Dallman et al, 1989).

## 7.8. SURFACE ANTIGEN EXPRESSION AND COLONIC CANCER

Colonic cancer cells have been shown to express various antigens on their surface. Some of these are weakly antigenic tumour associated antigens such as carcino-embryonic antigen (CEA). They also express a variety of histocompatibility antigens and blood group antigens. This expression appears to be variable and there is some evidence that tumour cells are capable of expressing non-self antigens.

### 7.8.1. Blood group antigens

Blood group antigens are a group of carbohydrate structures which can be identified on erythrocyte, endothelium, epithelium, and body secretions.

The main blood group antigens are known as A, B, and H. These antigens are glycolipids and glycoproteins which are found on the cell surface. The application of monoclonal antibodies to this field has boosted research into the potential roles of these carbohydrate antigens in cancer (Feizi, 1985).

In humans most blood group antigens are expressed throughout the fetal colon, but are absent from the distal colon in the adult (Denk et al, 1974; Cooper et al, 1978). Yuan et al (1985) confirmed this using monoclonal antibodies directed against the various blood group antigens. They also demonstrated enhanced distal expression of antigen in the distal colon of patients with colorectal cancer. These findings confirmed the oncofetal gene nature of blood group

antigens in bowel cancer.

One of the other important findings of this study was the high incidence of incompatible blood group antigen expression in cancer tissue. This was not seen in normal and fetal colonic tissue. This suggests that this is a cancer related phenomenon rather than being due to technical reasons (cross-reacting antibodies, for example). However, Schoentag et al (1987) were unable to demonstrate inappropriate blood group expression in a much larger series of 68 patients with colorectal cancer. They also found that metastatic tumour cells tended to have similar blood group antigen expression to the cells derived from the primary tumour. Other workers have provided evidence that some epithelial tumours of O and B patients express genetically inappropriate A antigen specificities (Lloyd, 1987).

#### 7.8.2. Major histocompatibility antigens

Daar et al (1983) demonstrated that HLA-DR antigens are expressed heterogenously on malignant colonic epithelium.

More recently, Moore et al (1986) analysed the major histocompatibility antigen status of epithelial cells from patients with colorectal neoplasms. They demonstrated that class I antigens were generally expressed on all normal and malignant epithelia. However, class II antigen expression was less marked with 30% of primary colorectal cancers having lost the capacity for class II

antigen expression. The process which stimulates expression of class II antigens is unknown at present.

Our present knowledge does not yet allow us to suggest possible immunological effects caused by differences in major (or minor) histocompatibility antigen expression between the host tumour and the transfused blood.

## 7.9. CURRENT TRANSFUSION PRACTICE

### 7.9.1. Surgery and anaesthesia

The insistence of some doctors that a patient for operation must have a haemoglobin in the normal range is reputed to have been described by Wintrobe as a form of superstition (Raven, 1981).

Fellin and Murphy (1987) reviewed the problems associated with preoperative anaemia. Under normal conditions oxygen extraction is only 25-30% and because oxygen transport depends on a variety of factors such as oxygen saturation of haemoglobin and cardiac output, anaemia alone may not decrease oxygen delivery to tissues. They posed the question, "What is a safe level of haemoglobin...?". Although many authors suggested a minimum haemoglobin level of 9 to 10 g/dl there little data to support this concept. On the contrary several studies had indicated that there was no increased risk associated with haemoglobin values between 5 and 10 g/dl, provided blood volume was normal. It was recognised that reduced blood volume did increase risk.

Anaemia probably does not affect wound healing. Three studies of wound dehiscence of abdominal wounds did not find anaemia to be a major contributing factor (Alexander et al, 1966; Mann et al, 1962; Marsh et al, 1954). Guiney et al (1966) found that 50% of patients with disrupted abdominal wounds had a haemoglobin of less than 12 g/dl, compared with 20% in the group of patients whose wounds healed

normally. Using a rabbit model, Heughan et al (1974) demonstrated that no significant change occurred in the oxygenation of experimental wounds in rabbits made anaemic by bleeding and retransfusing the separated plasma. In fact, in their model connective tissue synthesis was slightly greater at a haematocrit of 30% compared with a haematocrit of 40%. A rat model was used more recently by Foster et al (1985) to study the effect of hypovolaemia on healing of colonic anastomoses. They found that acute intraoperative loss of 10% circulating blood volume significantly impaired collagen concentration in bowel anastomoses although early anastomotic strength was not affected.

Lavies et al, (1985) discussed the short term hazards of not giving blood. They felt that the harmful effects of postoperative anaemia were difficult to prove. One of the main compensatory mechanisms for postoperative anaemia is an increased cardiac output. Clearly, the population of patients with colorectal cancer are elderly and such a compensatory mechanism may be inadequate and cause heart failure to varying degrees. They concluded that autotransfusion either with blood donated preoperatively or normovolaemic haemodilution at the start of surgery may be considered and improved surgical technique and greater use of epidural or spinal anaesthesia may help. They also suggested that postoperative oxygen therapy might be indicated for much longer periods than at present.

In a study of policies of preoperative and postoperative blood transfusion in patients undergoing colorectal resections in Wales, it was found that 56% of consultant surgeons would transfuse



patients who had a preoperative haemoglobin of less than 11 g/dl and 18% would transfuse with a haemoglobin of 11 g/dl (Foster and Ross, 1988). Given a preoperative haemoglobin of 12 g/dl, 51% of surgeons would transfuse if the haemoglobin fell to 9 g/dl or less. This suggested to the investigators that surgeons are over-transfusing their patients, particularly as a large proportion of surgeons arbitrarily transfuse preoperatively with no evidence of benefit.

Furthermore, it was considered that transfusion was unnecessary in 42% of transfused patients in a study of 476 patients who underwent surgery for colorectal cancer (Kiff and Kingston, 1988).

Transfusion was only considered necessary if the haemoglobin was less than 10g/dL. Of patients who were transfused only one unit of blood, 89% had a haemoglobin level of greater than 10g/dL.

Spence et al studied the cases of 107 Jehovah's Witnesses who had undergone major elective surgery (Spence et al, 1990). They concluded that mortality in elective surgery appeared to depend more on estimated blood loss than on preoperative haemoglobin levels, and that elective surgery could be performed safely in patients with a preoperative haemoglobin level as low as 6g/dL provided the estimated blood loss was less than 500mL.

#### 7.9.2. Blood products

During the early 1970s blood transfusion services were developing methods for preparing various blood components. The main advance

was the use of plastic blood containers which allowed component separation to be performed under sterile conditions within closed systems. In 1975 in England and Wales 90% of blood issued to clinicians consisted of whole blood (Gunson et al, 1986). Red cell concentrates (blood prepared by the removal of plasma) are now the main type of blood issued. Currently, it is generally recommended that red cell concentrates are the product of choice to treat patients with blood loss not exceeding 20-30%. Thus, two or three units of red cell concentrate are given with crystalloid solution to make up volume. For patients with loss of blood volume exceeding 20-30% further volume replacement is made with whole blood which contains coagulation factors.

## 7.10. AUTOLOGOUS TRANSFUSION

In patients undergoing surgery the main purpose of blood transfusion is to replace blood lost during the operation. Transfusion with autologous blood is an alternative to homologous blood. Autologous blood may be withdrawn from the patient preoperatively, or it may be transfused as it is shed.

One of the earliest examples of autotransfusion was described by Duncan in 1886. He reported a case of lower limb trauma as a result of a railway injury. The injured leg required amputation. However, a large amount of blood was lost during the patient's transfer from Kirkcaldy to the Royal Infirmary of Edinburgh. The patient displayed the clinical signs of hypovolaemic shock; "he was pallid and collapsed, with a pulse, when perceptible, quick, irregular and fluttering". Alcohol, ether injection, and elevation of the limbs failed to alleviate the situation. Intravenous injection of blood was indicated. Apparently, blood donors were readily obtainable in this large teaching hospital during the day, but not at night. During the subsequent amputation, blood was caught in a dish containing a solution of phosphate of soda. This was injected into the femoral vein of the stump. The patient recovered.

Apparently, intraoperative autotransfusion was not accepted into clinical practice until 1921 when its use in a patient with a cerebellar tumour was reported (Grant, 1921).

#### 7.10.1. Intraoperative autotransfusion

More recent attempts at intraoperative autotransfusion have met with problems including haemolysis, coagulopathy, emboli, renal failure and pulmonary problems (McShane et al, 1987). There have also been problems associated with the collection and washing of red cells collected during surgery. There are now specifically designed devices which surmount these problems.

McShane et al (1987) described one such device which was used with patients undergoing cardiac surgery involving cardiopulmonary bypass. The quality of blood salvaged at operation was compared with that of homologous donor blood. The saved blood had a higher haemoglobin concentration, a higher white cell count, a higher pH and a more physiological concentration of potassium than donated blood. This device produced good quality red blood cells of benefit to the patients. However, the saved blood had a high white cell count consisting almost entirely of neutrophils of which three quarters were damaged. It was suggested that these damaged white cells may release enzymes and contribute to the severity of adult respiratory distress syndrome in which leukocytes are thought to have an etiological role. The authors indicated that additional washing and centrifugation of collected blood might reduce this potential hazard.

In cancer patients intraoperative autotransfusion could have the risk of disseminating tumour cells. Klimberg, et al, (1986) reported a series of patients undergoing open surgery for kidney,

bladder and prostate tumours. Only five of these patients subsequently developed metastatic disease and this was thought to be a low incidence for these types of patients. These findings do not support the theory that intraoperative autotransfusion increases the risk of tumour dissemination. However, there are now specially designed filtering devices which can remove large cells including leukocytes and tumour cells from the blood prior to transfusion back into the patient.

Autologous blood deposit was described in a series of 235 cancer patients (Lichtiger et al, 1990). These patients underwent a variety of operations for a number of cancers (gastrointestinal, adrenal, genitourinary, bone and other sarcomas). Autologous blood was given to 77% of these patients without apparent sequelae.

Intraoperative autotransfusion is feasible in cancer patients, but perhaps patients undergoing surgery for bowel cancer would be at risk of dissemination of peritoneal bacteria. In elective colonic resection this risk is presumably low until the bowel is divided. However, in emergency colonic surgery the risk of peritoneal contamination will be higher and probably prohibitive. In elective cases there is an opportunity to withdraw autologous blood preoperatively and the need for intraoperative retrieval of blood will be minimal. This is not the case in emergency surgery and so intraoperative autotransfusion will probably have little to do with colonic surgery in the future.

#### 7.10.2. Autologous transfusion for elective surgery

Where elective surgery is planned in advance, the patient may attend the hospital two to three weeks prior to the proposed date of their operation. At this point one unit (500 ml) of blood is removed by venesection and stored in a conventional blood bank. A second unit of blood may be removed prior to surgery. Such a regime would clearly reduce the need for donated blood.

Nicholls et al (1986) reported a large study of autotransfusion in 336 patients. Most of these patients were undergoing orthopaedic and vascular surgery. The mean age for men in this group was 65 years and 63 years for women. In the larger group of men, the haemoglobin concentration fell by 1.2 g/dl to 14.1 g/dl after donation of one unit of blood and to 13.3 g/dl after two units. After venesection supplemental iron was given to encourage a brisk reticulocytosis. Patients in this programme were excluded if they were older than 75 years or if they were considered anaemic (haemoglobin less than 14 g/dl in men, and less than 13 g/dl in women).

The amount of blood not transfused seemed to depend on the operation performed. For patients having a total hip replacement and patients having a repair of abdominal aortic aneurysm, virtually all of the precollected blood was used. In lower limb vascular reconstruction only about 10% of autologous blood was used. A similar scheme for patients undergoing joint replacement was reported by James and Smith (1987).

This method of autologous transfusion could be applied to colorectal cancer patients. However, one would have to lower the haemoglobin level at which patients were excluded. In the retrospective study reported in this thesis, colorectal cancer patients who actually received a blood transfusion had a preoperative haemoglobin of around 12 g/dl. Preoperative venesection of two units of blood would lower the haemoglobin level to around 10 g/dl. At present this may not be acceptable to anaesthetists. The second problem would be the extra two week delay required to gather autologous blood. It is probable that the delay could be reduced by including blood donation in the lag between decision to operate and subsequent admission to hospital. In any case, the risks associated with such a delay are probably much less than those detrimental risks which are associated with blood transfusion.

Preoperative venesection is obviously not feasible in patients admitted for emergency surgery. This group of patients will continue to depend on donated homologous blood.

## 7.11. IMMUNOMODULATION

At the present time blood transfusion cannot be avoided in certain cases. If blood transfusion does cause immunosuppression in cancer patients, then is it possible to reverse this effect? Clearly this would involve altering the immune response to tumour cells. This process has been given the term of immunomodulation or immunotherapy.

Immunotherapy for cancer patients is not a new idea and the history of immunotherapy has been well reviewed by Priestman (1982). Cole and Humphrey (1985) recognised that immunosuppression is produced by major cancer operations and that this type of patient requires immunological help. They reviewed work that had been carried out in this field. However, this work probably did not take into account the more recent possibility of transfusion induced immunosuppression, but it is clear that generally increased immunosuppression in cancer patients correlates with an increasingly poor prognosis. Various nonspecific immune stimulators, such as BCG and *Corynebacterium parvum*, have in some trials seem to produce a small advantage for the cancer patient, but other studies have been less convincing.

Levamisole was one of the more promising agents (Amery et al, 1977), but this drug has untoward side effects. Windle et al (1987) found that a short postoperative course of levamisole together with a longer course of 5-fluoruracil reduced recurrence rates in patients



with colorectal cancer. This particular trial was stopped at an early stage because the early results suggested a possible detrimental effect on survival due to levamisole, although final analysis did not confirm this.

Trauma, shock, sepsis, and blood transfusion are usually associated with a degree of immunosuppression. The mechanisms remain unclear, but much attention is currently being directed at the ability of the macrophage to cause suppression of cell-mediated immunity locally by increased synthesis of arachidonic acid metabolites (particularly prostaglandin E<sub>2</sub>). It has been debated whether, or not, it is safe to commence clinical trials with prostaglandin synthetase inhibitors to attempt to reverse these changes. Of these drugs indomethacin would appear to be the safest. One compelling argument against the introduction of this therapy is that we do not know what happens if prostaglandin synthesis is stopped completely. The beneficial effect may only be gained with relatively low doses of indomethacin and moderate doses may be toxic in subjects with severe injury. In animals there is some unpublished evidence that splenomegaly results after treatment with indomethacin and this may represent a state of over stimulation.

However, indomethacin has been used clinically in the past. Panje (1981) reported seven cases of patients with head and neck cancer which demonstrated that indomethacin taken in standard dosages caused regression and stabilization of the disease. In patients who had received radiotherapy for head and neck cancer, it has been found that indomethacin enhanced lymphoblast transformation in

response to antigen (Maca et al, 1982).

Recent advances such as monoclonal antibodies, gene cloning, and cell cloning in relation to tumour immunology have been reviewed by Balch (1986). It is envisaged that these techniques will allow the complex immune mechanisms to be elucidated. Clinical trials involving specific rather than general immunomodulation will probably evolve in the years to come.

### Experimental

The experimental research described in this thesis raises several questions. Older rats seem to be susceptible to immunomodulation as a result of blood transfusion whereas younger rats are not. The influence of age needs to be studied further using other indicators of immune function as the true significance of peripheral lymphocyte population changes remains unclear.

The carcinogenesis model described will be useful, but it requires independent validation and must involve larger numbers of animals to confirm the abrogation of the transfusion effect with indomethacin.

Blood transfusion-induced immunosuppression acting via a macrophage/prostaglandin pathway is emerging as one of the possible explanations of the detrimental effects associated with transfusion in cancer patients. If this mechanism is responsible and it can be reversed using prostaglandin synthetase inhibitors such as indomethacin, then clinical trials will ensue. Before this can happen the influence of prostaglandin synthetase inhibitors on other cytokines and processes such as anastomotic healing must be demonstrated experimentally.

## Clinical

To clarify the immunological effects of blood transfusion in patients undergoing resection of colorectal cancer two types of studies should be undertaken.

Firstly, the immune function must be studied during the perioperative period and secondly, the influence of transfusion on the survival of patients after curative surgery should be determined.

In both studies randomisation will produce two groups of patients which are similar when the known major prognostic variables such as age, sex, site and stage of the tumour, are compared. One of these groups will receive blood transfusion according to standard practice and this transfusion will probably consist of whole blood and red cell concentrates. The second group must receive some form of blood volume replacement.

If blood volume replacement consists of plasma expander, then ethical considerations arise. Ethical committees will have to decide whether or not it is safe to correct significant blood loss without transfusing blood. If such a regime is considered to be safe, then it is conceivable that valid objections may be raised to the "unnecessary" transfusion of patients and exposing them to the many known hazards of blood transfusion such as transmission of viral infection. For similar reasons it may be difficult to recruit patients to such a trial. Furthermore, increased awareness

amongst medical staff involved in the trial may result in a more conservative transfusion practice which will diminish the number of patients who actually receive blood. Lastly, this type of trial will produce three groups of patients: one group will receive no blood, the second group would receive an amount of blood (say one to three units) or a similar volume of plasma expander, and the third group would consist of patients who received four or more units of blood and patients who were initially randomised to receive plasma expander, but who required subsequent transfusion with blood.

Clearly, the second group of patients would contain two subsets with similar tumours which could be compared. The third group which required relatively large transfusions could not be included in the survivorship studies. Blood volume replacement could also consist of autologous blood, but the numerical problems would still apply.

The alternative trial would compare two transfusion regimes. The first would consist of conventional blood while the second would consist of washed and filtered blood. This latter process would remove the antigen bearing components of blood: the white blood cells and the platelets. The two blood products could be randomised before delivery to the ward or operating theatre. Thus, medical staff would be truly blind as to which blood product was being given. The third group in the previous trial could then be included in the survivorship studies. This would reduce the number of patients required to achieve a statistically significant result. The disadvantages of this trial include the financial and logistic implications for the blood transfusion services. However, widespread adoption of pretransplant transfusion for renal

transplant patients was based on retrospective and experimental studies and not on randomised, prospective clinical trials. Similarly, there may be a demand for blood free of histocompatibility antigen and in the long-term it would be in the interests of the blood transfusion services to invest in trials in order to ascertain the this type of blood is in fact advantageous to the patients.

This thesis presents clinical evidence which suggests that blood transfusion is associated with decreased survival in patients with colorectal cancer. However, blood transfusion possibly represents an index of disease severity and current multicentre prospective studies will hopefully address this problem.

In the Sprague Dawley rat, allogeneic blood transfusion was not associated with changes in peripheral T lymphocyte subsets in normal young rats. In older rats blood transfusion resulted in an increase in the proportion of OX 8 T lymphocytes. This suggests that older subjects may be more susceptible to the immunomodulatory effects of blood transfusion, although it is emphasised that this work did not include functional assays of the T cell subsets.

Allogeneic blood transfusion enhanced colonic carcinogenesis in rats and treatment with indomethacin during the period of transfusion may reverse this effect.

Rat peritoneal macrophage prostaglandin E<sub>2</sub> was increased by blood transfusion and this appeared to be related to storage conditions of blood and was seen in both syngeneic and allogeneic blood in addition to genetic factors. Although prostaglandin E<sub>2</sub> has been shown to have many immunosuppressive effects, one can only guess the role it plays in transfusion-mediated immunosuppression. It must be established what effects blood transfusion has on other macrophage products, eg: tumour necrosis factor, superoxide and interleukin 1.

On reviewing the many retrospective studies it was found that many did not control for variables related to blood transfusion which could affect disease recurrence (blood loss, duration of anaesthesia and surgery in particular). Overall, there is little agreement regarding the significance of blood transfusion in patients with colorectal cancer. Therefore, on the basis of these retrospective studies, one cannot recommend changes in transfusion practice. However, there is now increasing prospective clinical evidence to suggest that blood transfusion may indeed have an immunosuppressive effect in cancer patients.

Autologous blood transfusion appears to be safe in cancer patients and stricter transfusion regimes may reduce the number of patients who receive allogeneic blood. The role of erythropoietin is yet to be established. There will remain a proportion of patients who will continue to require allogeneic transfusion either because they present as an emergency, or because haemorrhage is severe. Blood products containing large amounts of plasma may have the strongest immunosuppressive effects.

Pharmacological immunomodulation, possibly using prostaglandin synthetase inhibitors, may be required to abrogate the detrimental effects of blood transfusion on the immune response.



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## BLOOD TRANSFUSION AND COLONIC CANCER IN THE RAT

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Blood transfusion (BT) may cause immunosuppression which could have a detrimental effect in cancer patients. Previous experimental cancer models have used transplanted tumour cells rather than an autochthonous tumour model. The aim of this research was to assess the effects of allogeneic blood transfusion on chemically induced colonic carcinogenesis in rats. At 28 weeks after commencing carcinogenesis with dimethylhydrazine rats transfused with allogeneic blood ( $n = 26$ ) had a mean of 6.0 (SD = 2.7) macroscopic tumours per rat and rats transfused with NaCl ( $n = 23$ ) had a mean of 4.1 (SD = 2.7) tumours per rat;  $p = 0.02$ . Some of these tumours arose in close proximity and converged, making it impossible to state that they were truly separate tumours. It is suggested that a lower dose of dimethylhydrazine is used to reduce the extent of cancer and that large numbers of rats may be required to achieve more highly significant results.

**KEY WORDS:** Colon cancer, carcinogenesis, blood transfusion, T lymphocytes.

### INTRODUCTION

Blood transfusion (BT) may cause immunosuppression which could have a detrimental effect in burns and cancer patients.<sup>1</sup> Previous experimental cancer models have used transplanted tumour cells rather than an autochthonous tumour model. The aim of this research was to assess the effects of allogeneic blood transfusion on chemically induced colonic carcinogenesis in rats.

### MATERIALS AND METHODS

#### *Carcinogenesis*

The DMH was prepared in a concentration of 10 mg/ml in 1 litre of 0.9% NaCl. To this was added 15 g of ethylene-diaminetetracetate (EDTA). This was done to prevent dehydrogenation which occurs rapidly in the presence of trace metals. The pH of the solution was brought to pH 7.0 with 1 M Na<sub>2</sub>CO<sub>3</sub>.

Male Sprague Dawley rats were used throughout the experiments. They were fed on a standard pellet diet and tap water ad libitum. A group of 62 ten week old rats were given 16 weekly injections of dimethylhydrazine (DMH) at a dose of 16 mg/kg.<sup>2</sup> After the eighth injection rats were transfused with either allogeneic blood or NaCl.

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These rats were killed at 28 weeks and tumour development assessed. A further group of 22 rats also treated with DMH were killed at 36 weeks. A third group of 29 rats were not treated with DMH and these were randomised at eight weeks to receive transfusions of blood or NaCl.

#### *Assessment of Carcinogenesis*

The rats were killed at the appropriate time by ether anaesthesia. The animals were weighed and inspected for external signs of pathology. The most obvious signs were related to the development of tumours of the external auditory canal. A careful search was made of the visceral and parietal peritoneum for metastatic deposits. The small bowel was removed whole and placed in 0.9% NaCl. The liver and lungs were removed and weighed and sliced in sections to look for hepatic metastases. The large bowel was opened longitudinally and faecal material was washed off with fresh saline. The length of large bowel, which measured about 30 cm, was placed on a metal rule. The length was noted. The position and diameters of each macroscopic tumour were noted. Many tumours were related to the lymphoid patch found in the wall of the rectum and diagrams were made of these tumours. The diameters were measured using calipers. Magnification was obtained using a 1.5 binocular loupe. The large bowel was then wrapped like a swiss roll on a wooden rod and placed in 10% formalin (phosphate buffered) solution. Histological slides were prepared and stained with haematoxylin and eosin. The tumours were assessed and graded by an independent pathologist.

#### *T Lymphocyte Subset Analysis*

Animals were anaesthetised with ether and blood withdrawn from the tail vein. Ficoll-Hypaque density gradient columns were used to separate the lymphocytes. The interface between the Ficoll Hypaque and plasma contains monocytes and lymphocytes and this interface was removed. These cells were washed twice in fresh RPMI and the cells suspended at  $4 \times 10^6$ /ml. A portion of the isolated cells was used to prepare a slide for differential analysis, using a centrifuge to assess the purity of the lymphocyte preparation. Four samples were then prepared. In one of these tubes was placed 2 ml of PBS/10% fetal calf serum (FCS) to which 400  $\mu$ l of the cell suspension were added. The tube was centrifuged at 1500 rpm for 10 minutes at 4°C. The supernatant was discarded and 400  $\mu$ l of PBS/10% FCS was added to the cell pellet. The cells were resuspended and 100  $\mu$ l of this suspension were transferred into each of the three remaining test tubes which contained the monoclonal antibodies. The monoclonal antibodies used were: W3/13 for total T lymphocytes, W3/25 for helper T lymphocytes and OX8 for suppressor/cytotoxic lymphocytes.<sup>3</sup>

The cells were then incubated with fluorescein isothiocyanate conjugated anti mouse IgG. Flow cytometric analysis was used to analyse the T lymphocyte subsets. The Becton Dickinson FACS 440 was used. For each sample of labelled cells the machine counted 5000 lymphocytes. It counted the number of these cells which fluoresced, therefore the proportion of labelled cells could be calculated.

#### *Blood Transfusion*

Male DA rats were used as blood donors throughout the series of experiments. These rats were normal and had not been used in previous experiments and had not

previously been used as blood donors. The donor animals were anaesthetised with ether and the thorax swabbed with chlorhexidine solution. Blood was withdrawn by cardiac puncture into a syringe containing a small amount of heparin to prevent the blood clotting. The concentration of heparin used was about 30 units/ml. This blood was transfused to the recipients within one hour. The recipient rats were anaesthetised lightly with ether. The volume injected was 1.5 ml in all cases. Three attempts were made to enter the tail vein and if venepuncture was unsuccessful a cut-down was performed on the jugular vein.

### Statistics

The results of the T lymphocyte subset analysis are the mean (standard deviation) of fluorescent cells expressed as the proportion of the total cells counted (5000). The paired t-test formula was used to compare the differences between the mean proportions.

## RESULTS

Allogeneic blood transfusion (compared with NaCl transfusion) did not alter T lymphocyte subsets either at one week or at 18 weeks after transfusion (Tables I and

TABLE I

Effect of allogeneic blood or NaCl transfusion on T lymphocytes subsets in 6-week-old rats which have been treated with dimethylhydrazine — paired data

Monoclonal antibody	mean % cells labelled (SD)		
	W3/13	W3/25	OX8
A. Blood			
pre-transfusion, n = 17	65.8 (10.6)	27.2 (8.0)	35.2 (7.61)
post-transfusion	68.0 (9.1)	24.2 (8.7)	33.9 (9.7)
p value: t test	0.51	0.30	0.67
B. NaCl			
pre-transfusion, n = 14	69.0 (10.6)	29.9 (12.4)	34.4 (9.3)
post-transfusion	63.3 (10.6)	21.5 (8.7)	35.9 (12.9)
p value: t test	0.12	0.024	0.69

TABLE II

Comparison of peripheral blood lymphocytes at 30 weeks

Monoclonal antibody	Mean % cells labelled (SD)		
	W3/13	W3/25	OX8
Transfusion group			
A. DMH-treated rats			
blood, n = 26	66.3 (11.0)	34.9 (9.4)	30.3 (8.0)
saline, n = 23	67.8 (11.1)	35.2 (11.0)	32.0 (9.1)
p value: t test	0.63	0.90	0.50
B. untreated rats			
blood, n = 6	63.7 (8.1)	30.7 (11.5)	37.3 (6.3)
saline, n = 9	71.6 (14.4)	25.0 (5.8)	40.1 (12.4)
p value: t test	0.26	0.23	0.62

II). None of untreated rats developed tumours. All rats treated with DMH had developed colonic malignancy and some developed ear and small bowel tumours (Table III). The second set of 22 rats treated with dimethylhydrazine and had identical transfusions to the first and second sets. This third group was killed at 34 weeks. By this time 2/11 rats transfused with blood had died due to malignancy and 4/11 rats transfused with 0.9% saline had died due to malignancy.

At 28 weeks rats transfused with blood ( $n = 26$ ) had a mean of 6.0 (SD = 2.7) macroscopic tumours per rat and rats transfused with NaCl ( $n = 23$ ) had a mean of 4.1 (SD = 2.7) tumours per rat;  $p = 0.02$ . The specimens were analysed by an independent pathologist and histological features noted (Table IV). In some adjacent tumours, it was not possible to identify them as histologically separate. The second group of DMH-treated rats given BT ( $n = 9$ ) and rats given NaCl ( $n = 7$ ) killed at 36 weeks had advanced malignancy, but had similar numbers of bowel tumours; 9.0 (SD = 4.8) versus 10.6 (SD = 2.9);  $p = 0.5$ .

## DISCUSSION

There are now several retrospective clinical studies which suggest that in patients with colorectal cancer, the receipt of perioperative blood transfusion is associated with a poorer prognosis. Other studies fail to demonstrate a statistically significant result, but present a clear trend which clearly suggests that transfused patients fare less well. These studies have been reviewed.<sup>1</sup> It is possible that transfused patients lost more blood because of the severity of their malignant disease, despite having, sanguinely,

TABLE III

Dimethylhydrazine carcinogenesis in Sprague Dawley rats transfused with allogeneic blood or 0.9% saline. comparison of macroscopic tumour distribution

	blood	saline	p*
tumour			
colonic	26/26	23/23	NS
small bowel	3/26	7/23	NS
metastatic	7/26	7/23	NS
ear	5/26	5/23	NS

\*chi squared test.

TABLE IV

Dimethylhydrazine carcinogenesis in Sprague Dawley rats transfused with allogeneic blood or 0.9% saline. comparison of histopathologic features

Transfusion:	blood $n = 26$	saline $n = 23$	p*
adenoma; n, %	30, 27%	23, 29%	N.S.
carcinoma; n, %	79, 73%	55, 71%	N.S.
histological grade			
G1	14, 17%	12, 22%	N.S.
G2	39, 51%	28, 51%	N.S.
G3	26, 32%	15, 27%	N.S.
mucin production	54, 70%	32, 58%	N.S.
lymphocyte infiltration	39, 49%	24, 44%	N.S.

had a curative, though sanguinous, resection. This conundrum will only be solved by the multicentre clinical trials which are now underway.

Experimental work on the effects of blood transfusion on tumour behaviour yields conflicting conclusions. In 1954, George Davies Snell reported that pretreatment with some tissue preparations could produce tolerance of tumour transplants in host mice. This effect could be produced by allogeneic blood transfusion in a sarcoma transplant model.<sup>4,5</sup> However, since then various groups have used other animal models to test the effect of blood transfusion on tumour growth.<sup>6-15</sup> Using an athymic mouse model Francis *et al.* demonstrated that blood transfusion enhance the growth of transplanted melanoma cells and that this effect depended on the presence of the thymus gland and by implication that cell-mediated immunity must be involved in the mechanism.<sup>16</sup> A smaller number of studies present conflicting findings which suggest that blood transfusion does not enhance tumour growth.<sup>17-19</sup> There are also two studies which showed that blood transfusion decreased tumour growth, one of which showed that in mice allogeneic blood transfusion before and after inoculation with mammary cancer cells prolonged survival.<sup>20,21</sup>

All of the studies cited used transplanted tumour cells. We have used an experimental model which does not involve tumour transplantation. This autochthonous cancer model is closer to the human colonic cancer disease, but it has some disadvantages which could be remedied with modification. We conclude that blood transfusion enhances the growth of chemically induced colonic tumours rather than increasing the number of tumours produced. This effect does not appear to involve changes in T lymphocyte subpopulations in the peripheral blood and we, therefore, question the value of this test in assessing blood transfusion immunomodulation. However, this model could be used to investigate the modulation of the immunosuppressive effects of blood transfusion in colonic cancer. It is suggested that a lower dose of dimethylhydrazine is used to reduce the extent of cancer and that larger numbers of rats may be required to achieve more highly significant results.

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## BLOOD TRANSFUSION AND PROSTAGLANDIN PRODUCTION BY RAT PERITONEAL MACROPHAGES

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Allogeneic blood transfusion has been associated with suppression of cell-mediated immunity and this possible effect may be mediated by increased production of prostaglandins by macrophages. The effect of allogeneic blood transfusion on macrophage arachidonic acid metabolite production was studied in the Sprague Dawley rat. In this strain of rat we found that blood transfusion did not influence arachidonic acid metabolite eicosanoid when compared with rats transfused with saline, although there appeared to be a decrease associated with anaesthesia and surgery which may have masked the effects of blood transfusion. Furthermore, stimulation of macrophages with calcium ionophore again failed to demonstrate an increased prostaglandin production after blood transfusion — an effect which had been observed previously by others. These results suggest that the influence of allogeneic blood transfusion on arachidonic acid metabolites may be limited to specific strains of animal.

KEY WORDS: Blood transfusion, prostaglandins, Macrophages.

### INTRODUCTION

Blood transfusion may cause immunosuppression which could have a detrimental effect in burns and cancer patients. We have established a rat model to study the effect of allogeneic blood transfusion on the growth of chemically induced colonic tumours.<sup>1</sup> The mechanism of this effect remains unclear, but recently attention has focused on the theory suggested by Waymack who suggests that immunosuppression caused by blood transfusion is mediated by an increase in macrophage prostaglandin synthesis.<sup>2</sup> The aim of our research was to analyse peritoneal macrophage arachidonic acid synthesis using both the donor and recipient strains used in the rat colon carcinogenesis model.

### MATERIALS AND METHODS

In Experiment I male Sprague Dawley rats weighing 350–400 g were divided into two groups. Rats in group one ( $n = 4$ ) were not transfused and rats in group two ( $n = 5$ ) were given a single intravenous transfusion of allogeneic blood. This was fresh heparinised whole blood obtained by cardiac puncture from DA rats. A volume of

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1.5 ml was injected into the tail vein of the recipient. In Experiment II rats were divided into three groups. Group (n = 4) one were not transfused, Group 2 (n = 5) were transfused with 1.5 ml of heparinised 0.9% NaCl, and Group 3 (n = 4) were given one intravenous transfusion of allogeneic blood. Eight days after transfusion, the peritoneal macrophages were harvested. In Experiment I macrophage prostaglandin E2 synthesis was measured in resting cultured cells. In Experiment II, after incubation with various concentrations of the calcium ionophore A23187 (Cambridge Bioscience: Cambridge, UK), immunoreactive prostaglandin E2 (PGE2), and 6-keto-prostaglandin F1  $\alpha$ , thromboxane B2 (TXB2), and leukotriene C4 concentrations were estimated.

#### *Macrophage harvesting*

Four days after transfusion, the peritoneal cavity of each animal was infused with 3.4 ml of sterile brain-heart infusate (Oxford Ltd: Basingstoke, UK). The needle was introduced into the lower left quadrant of the abdomen in order to minimise the risk of perforating the caecum. Eight days after transfusion, the peritoneal macrophages were harvested.

The rats were anaesthetised by the open ether method. The abdomen was shaved and the area washed once with 100% ethanol. The skin of the abdominal wall was removed by sharp dissection. The peritoneal cavities were lavaged by injecting 10 ml of sterile Phosphate Buffered Saline (PBS) pH 7.3, containing 5 mmol ethylene-diaminetetracetate (EDTA). The abdomen was greatly massaged for 2 minutes to ensure dispersion of the lavage fluid. An incision was made in the flank of the animal on the right side. A sterile glass Pasteur pipette was used to collect the peritoneal fluid. Approximately 8 ml of fluid was collected each time.

#### *Preparation of the macrophages for culture*

The peritoneal fluid was centrifuged at 300 g for 10 minutes at 10°C. The supernatant was removed, and the cell pellet resuspended and washed twice in PBS. Contaminating red blood cells were lysed hypotonically by adding 2 ml 0.2% NaCl for 1 minute followed by 2 ml of 1.6% NaCl for 1 minute. The leukocytes were resuspended in PBS to a known volume and the cell number counted using a Coulter Counter (Coulter Electronics Ltd: Luton, UK).

The cells were resuspended in medium 199 with Earle's balanced salt solution, EBSS (Flow Laboratories: Herts. UK) supplemented with penicillin/streptomycin (Flow Laboratories) L-glutamine (Flow Laboratories) and 7.5% fetal calf serum (Flow Laboratories) to a final concentration of  $10^6$  cells per ml.

#### *Macrophage culture*

Two million cells were added to plastic tissue culture plates (Gibco Ltd: Uxbridge, UK) and they were incubated at 37°C in an atmosphere of 5% carbon dioxide for one hour. The culture medium was removed and replaced with fresh M-199. This process removed non-adherent cells leaving the adherent macrophages. The macrophage suspensions were then cultured for period of 18 hours. The entire medium was then removed and frozen at -70°C prior to assay.

*Quantitation of arachidonic acid metabolites*

Radioimmunoassays (RIA) were employed for measurement of cyclooxygenase products PGI<sub>2</sub> measured as 6-keto-PGF<sub>1</sub>  $\alpha$ , PGE<sub>2</sub> as well as for leukotriene LTC<sub>4</sub>. 100  $\mu$ l samples of macrophage supernatants, or of synthetic standard; 100  $\mu$ l of specific antibody and 100  $\mu$ l [<sup>3</sup>H]-eicosanoid (Amersham International: Cardiff, UK) were incubated in 3.5 ml polypropylene test tubes (Sarstedt: Leicester, UK) at 4°C for 18 hours. The non-protein bound eicosanoids were precipitated by the addition of 250  $\mu$ l of dextran (T70, Pharmacia: Uppsala, Sweden) coated charcoal, (Norit SX1, BDH Chemicals: Poole, UK), both 1% (w/v) in Tris-Isogel buffer, followed by centrifugation at 250 g for 10 minutes, 4°C. Supernatants were decanted and mixed with 3.5 ml of optiphase MP scintillant (LKB Instruments: Poole, UK) and the radioactivity measured in a Rackbeta liquid scintillation counter (LKB: Turku, Finland).

The synthetic and measured eicosanoids were detected in the linear portion of the radioligand binding curve. Synthetically prepared prostaglandin standards were obtained from Sigma Ltd: Poole, UK. Leukotriene standard was also synthetically prepared and was a kind gift by Dr Brendt Spur.

*Statistics*

The t-test was used to compare the mean metabolite concentrations in the various experimental groups.

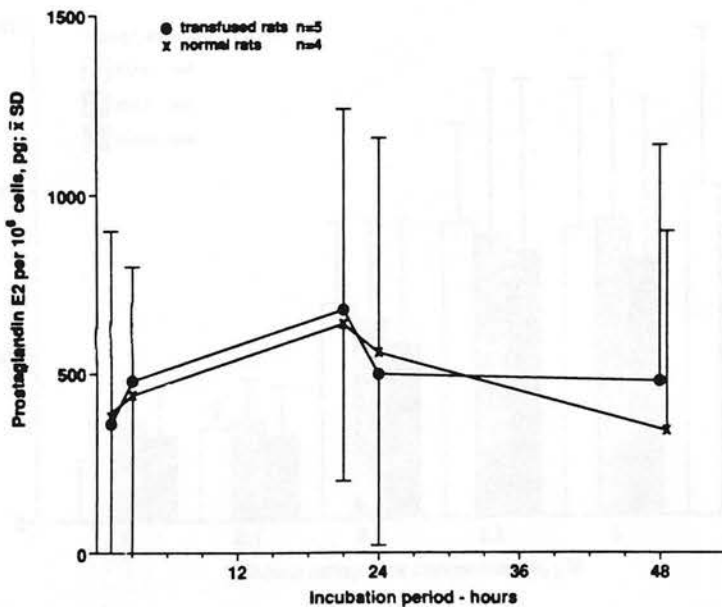


FIGURE 1 PGE<sub>2</sub> production by resting rat peritoneal macrophages in culture.



RESULTS

*Time Course Experiments of PGE2 Release*

PGE2 was generated by resting cultured peritoneal macrophages in a time dependent manner reaching a mean maximum after 19 hours of incubation of  $658 \pm 445\text{pg}/10^6$  cells (Figure 1). The release of PGE2 by macrophages from transfused animals followed a similar pattern and was no different in quantitative terms or in comparison of the kinetics of generation.

*Arachidonic Acid Metabolism Following Ca Ionophore Stimulation*

The results of calcium ionophore stimulation are shown in Figures 2–5. The results show that maximal stimulation of thromboxane B2 and 6 keto prostaglandin F1 alpha synthesis is reached with a calcium ionophore concentration of  $0.5\text{ }\mu\text{M}$ , while maximal synthesis of prostaglandin E2 and leukotriene C4 is achieved with calcium ionophore of  $2.5\text{ }\mu\text{M}$ . In all cases increasing calcium ionophore concentration above these levels did not increase synthesis. There were no significant differences between the three groups when prostaglandin E2 was measured. When thromboxane B2 was assayed the synthesis of this was slightly reduced after transfusion with either saline or blood, although this did not reach statistical significance. A similar effect was seen after maximal stimulation of prostaglandin F1 alpha and leukotriene C4, the latter reaching statistical significance at calcium ionophore concentrations greater than  $0.5\text{ }\mu\text{M}$  ( $p < 0.05$ ).

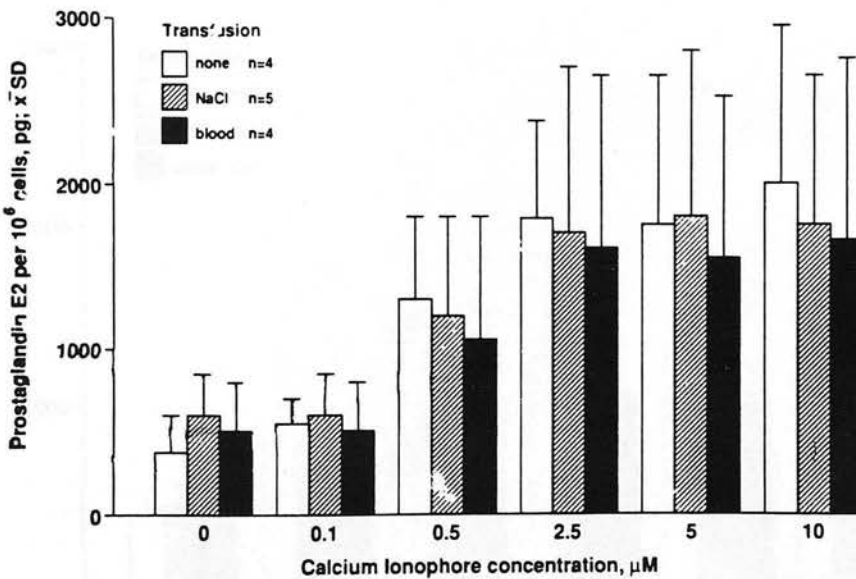


FIGURE 2 PGE2 production by stimulated rat peritoneal macrophages in culture.

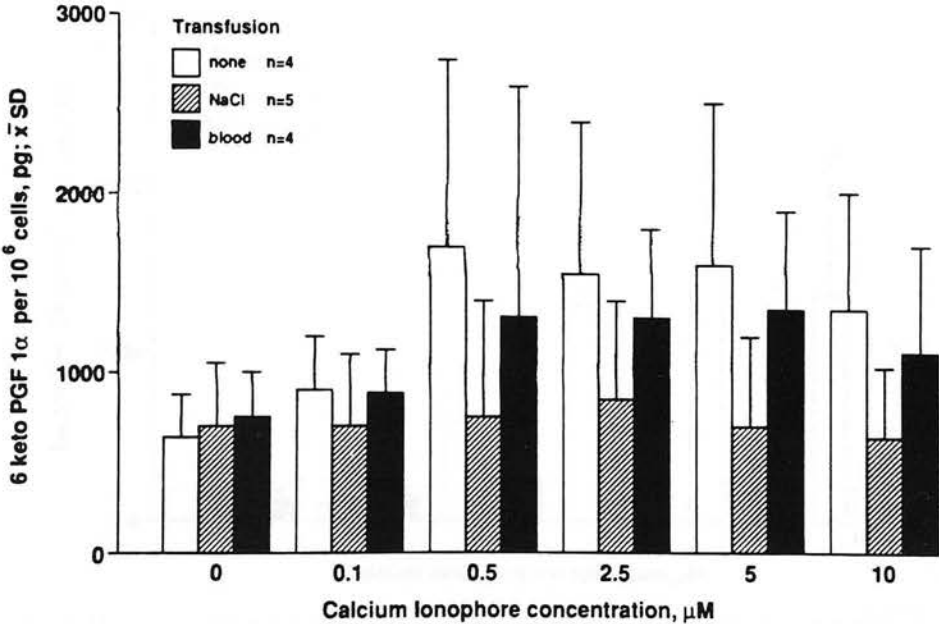


FIGURE 3 6-keto PGF1 $\alpha$  production by stimulated rat peritoneal macrophages in culture.

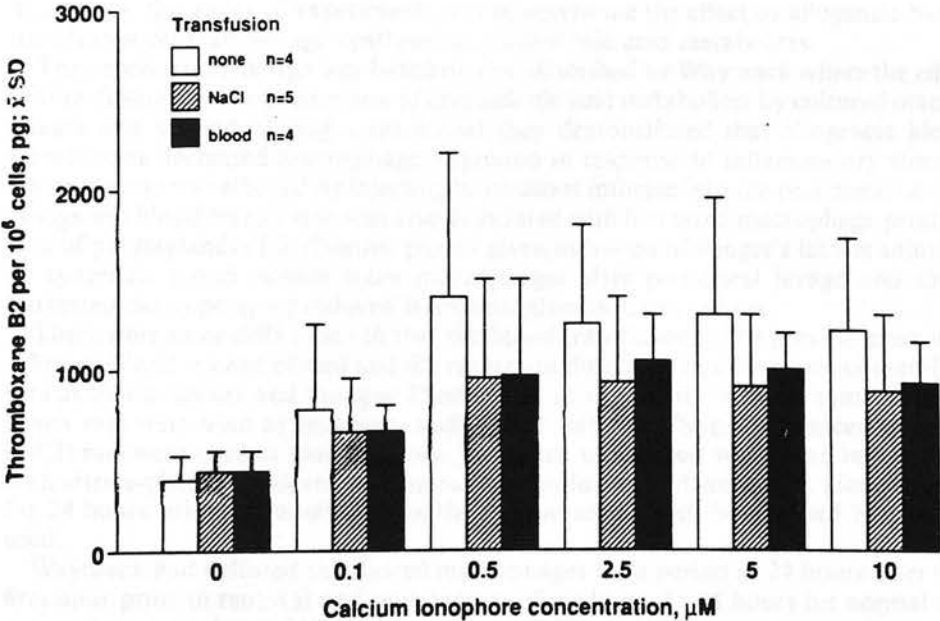


FIGURE 4 Thromboxane B2 production by stimulated rat peritoneal macrophages in culture.

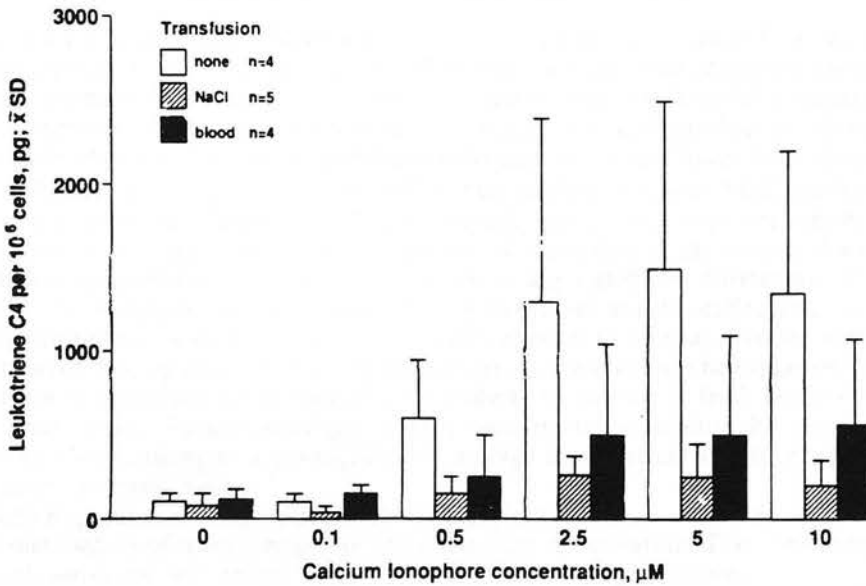


FIGURE 5 Leukotriene C4 production by stimulated rat peritoneal macrophages in culture.

## DISCUSSION

The aim of this series of experiments was to determine the effect of allogeneic blood transfusion on macrophage synthesis of arachidonic acid metabolites.

The experimental design was based on that described by Waymack where the effect of transfusions on the production of arachidonic acid metabolites by cultured macrophages was studied.<sup>2</sup> Using a rat model they demonstrated that allogeneic blood transfusions decreased macrophage migration in response to inflammatory stimuli. This response was elicited by injecting brain-heart infusate into the peritoneal cavity. Allogeneic blood transfusion was also associated with increased macrophage production of prostaglandin E2. Control groups given infusions of Ringer's lactate solution or syngeneic blood yielded more macrophages after peritoneal lavage and these harvested macrophages produced less prostaglandin E2 in culture.

There were some differences in that the blood transfusion in the present series was 1.5 ml of blood instead of 1 ml and the rat strains differed. The current series used DA rats as blood donors and Sprague Dawley rats as recipients. In the Waymack series Lewis rats were used as recipients and Buffalo rats or A'Sogaloff Cancer Institute (ACI) rats were used as blood donors. Waymack used blood which had been mixed with citrate-phosphate-dextrose anticoagulant solution and stored the blood at 4°C for 24 hours prior to transfusion. In the present series fresh heparinised blood was used.

Waymack had cultured the elicited macrophages for a period of 24 hours after the first hour prior to removal and replacement of medium. At 24 hours his normal rat macrophages produced 740 (SEM = 89) pg PGE per  $10^6$  macrophages. This compares favourably with the present results: 563 (SD = 584) pg per  $10^6$  macrophages. It will be seen that there is a marked degree of variation in results as expressed by the

large standard deviation. Waymack's series of experiments consisted of relatively large numbers of rats to achieve statistical results: the experimental groups contained 20 animals each. Waymack found a 40% increase in PGE synthesis after transfusion with syngeneic Lewis blood, but this did not reach statistical significance. However, they were able to demonstrate significant increases after transfusion with allogeneic blood. Transfusion with blood from buffalo rats resulted in similar PGE synthesis to that found after transfusion with ACI rat blood. This finding does not support the suggestion that a variation in results depends on the strain of rat used as donors.

The current series of experiments did not show any significant differences, or even trends, which support the theory proposed by Waymack and his colleagues, i.e. that immunosuppression seen following transfusion appears to be related to an increased synthesis of prostaglandin E. The reason for this discrepancy may be explained by the variation in experimental techniques (in particular by the use of fresh blood instead of stored blood). Future investigation may explore the possibility that changes in donated blood arising as a consequence of storage may account for its effect on the recipient's immune system.

Both Experiment I and those conducted by Waymack *et al.* measured arachidonic acid metabolites of macrophages at a baseline state of activation. The levels reported in both series are well below those found in inflammatory situations.

The aim of Experiment II was to assess the response of macrophages to stimulation with calcium ionophore and to determine any differences between normal untreated rats and rats transfused with either allogeneic blood or saline. The results show that the levels of arachidonic acid metabolites were lower if rats had been transfused with either NaCl or blood. This was particularly striking with leukotriene C4. This is difficult to explain, but the transfused rats underwent one additional anaesthetic with ether and a venepuncture to administer the transfusion. This suggests that macrophage synthesis of these metabolites is less readily stimulated in rats subjected to surgical trauma and repeated anaesthesia. In this situation, stimulation of prostaglandin F1 alpha and leukotriene C4 was least in the macrophages of rats transfused with saline. Reduced leukotriene production is thought to impair host defence mechanisms and increase the risk of bacterial infections.

Relatively small numbers of rats were included in the various experimental groups and it is suggested that future research will be required to verify these findings.

The macrophage is now known to have a key role in the regulation of cell-mediated immunity. Some of these effects are mediated by arachidonic acid metabolites secreted by the macrophage and this cell may play a central role in the immunosuppressive response to blood transfusion.

In conclusion, our experiments have failed to support the theory that allogeneic blood transfusion is associated with an increase of prostaglandin production by peritoneal macrophages in the rat. However, we have demonstrated that prostaglandin production by stimulated macrophages in this experimental model is influenced by operative trauma and/or ether anaesthesia.

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